CHARACTERIZATION OF ANTIBIOTIC RESISTANCE GENES FROM *MYCOBACTERIUM ABSCESSUS*

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To Mark ~

Τοῖς τολμῶσιν ἡ τύχη ξύμφορος". - *Θουκυδίδης*

"Fortune favours the bold". – Thucydides

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SUMMARY

Mycobacterium abscessus, a relative of Koch's bacillus, has recently emerged for an increasing number of disease manifestations, ranging from pulmonary to skin infections. Its high intrinsic antibiotic resistance poses a significant threat and often leads to its persistence when other pathogens have been successfully eradicated by antimicrobial chemotherapy. Indeed, *M. abscessus* is naturally resistant, not only to the conventional first-line anti-tuberculosis drugs, but also towards most major classes of antibiotics used for treatment of $Gram^+$ and $Gram^-$ bacterial infections, namely β-lactams, aminoglycosides and macrolides. This notoriety of *M. abscessus* has lately motivated several research groups worldwide to study this bacterium in order to decipher its antibiotic resistance mechanisms.

M. abscessus genome analyses revealed various genes putatively involved in its innate rifamycin, aminoglycoside and β-lactam resistance. However, the exact function and the molecular role of these genes on the pathogen's high degree of resistance remain under cover. As elucidation of drug resistance mechanisms, heavily relies on proper genetic manipulation and particularly, on generation of mutants by targeted gene inactivation, research attempts into *M. abscessus* were hampered by the limited number of available genetic tools.

Aiming to investigate the molecular basis underlying rifamycin, aminoglycoside and β-lactam resistance in *M. abscessus*, we firstly established an apramycin positive selection and a novel *katG*-dependent isoniazid counterselection strategy to generate deletion mutants of the putatively involved genes. Subsequently, we performed minimal inhibitory concentration (MIC) assays towards a variety of antimicrobial agents to determine the susceptibility level of the deletion mutants in comparison to the *M. abscessus* parental strain.

This study identified (i) Arr_*Mab*, a rifampicin ADP-ribosyltransferase, which ribosylates and thereby inactivates rifampicin, as the major determinant of innate rifamycin resistance in *M. abscessus*; (ii) AAC(2') and Eis2, two acetyltransferases which transfer acetyl groups on crucial residues of aminoglycoside drugs leading to their inactivation, as two distinct aminoglycoside resistance determinants which confer *M. abscessus* clinically relevant drug resistance; and (iii) Bla_*Mab*, a broad-spectrum class A β-lactamase, which rapidly hydrolyses most β-lactam antibiotics except imipenem, meropenem and cefoxitin, as the major determinant of innate β-lactam resistance in *M. abscessus.*

These findings build basic knowledge on *M. abscessus* intervention strategies and testify to the urgent need to develop novel compounds that can escape Arr_*Mab*-mediated rifamycin resistance, AAC(2')- and Eis2-mediated aminoglycoside resistance and Bla_*Mab*-mediated β-lactam resistance of this highly resistant pathogen.

ZUSAMMENFASSUNG

Mycobacterium abscessus ist in letzter Zeit immer öfter als Verursacher verschiedener Krankheitsbilder, von pulmonalen Erkrankungen bis hin zu Hautinfektionen, beschrieben worden. Seine hohe, intrinsische Antibiotikaresistenz stellt eine erhebliche Bedrohung dar, weil sie erheblich zur Persistenz des Erregers beiträgt. *M. abscessus* verfügt nicht nur über eine natürliche Resistenz gegenüber den konventionellen first-line Medikamenten gegen Tuberkulose, sondern auch gegenüber der Mehrheit der Antibiotika die zur Behandlung von $Gram^+$ und $Gram^-$ bakteriellen Infektionen eingesetzt werden, wie β-Laktamen, Aminoglykosiden und Makroliden. Diese Befunde haben jüngst weltweit Forschungsgruppen angeregt, *M. abscessus* zu studieren um die Mechanismen der Antibiotikaresistenz zu entschlüsseln.

Genom-Untersuchungen von *M. abscessus* identifizierten verschiedene Gene die vermutlich an der intrinsischen Resistenz gegenüber Rifampicin, Aminoglykosiden und β-Laktamen beteiligt sind. Die genaue Funktion und molekulare Bedeutung dieser Gene, blieb jedoch bislang verborgen. Die Aufklärung von Resistenzmechanismen basiert auf der Verfügbarkeit von geeigneten genetischen Werkzeugen, die zur gezielten Generierung von Deletionsmutanten verwendet werden können. Aufgrund der geringen Anzahl verfügbarer genetischer Werkzeuge waren die Forschungsbestrebungen für *M. abscessus* eingeschränkt.

Zur Untersuchung der molekularen Ursachen der Rifampicin-, Aminoglykosid- und β-Laktamresistenz in *M. abscessus*, etablierten wir zuerst eine Apramycin Positivselektion und eine neue *katG*-abhängige Isoniazid Gegenselektion, um Deletions-Mutanten der vermeintlich beteiligten Gene zu generieren. Anschliessend führten wir Untersuchungen zu Minimalen Inhibitionskonzentrationen verschiedener antimikrobieller Substanzen durch, um die Antibiotikaempfindlichkeit der Deletionsmutanten mit der Empfindlichkeit des ursprünglichen Erregerstammes von *M. abscessus* zu vergleichen.

Diese Arbeit identifizierte i) Arr_*Mab*, eine Rifampicin ADP-Ribosyltransferase, die Rifampicin eine Ribosylgruppe hinzufügt und es somit inaktiviert, als massgeblichen Faktor der intrinsischen Rifampicin Resistenz von *M. abscessus*; ii) AAC(2') und Eis2, zwei Acetyltransferasen, die Acetylgruppen an entscheidenden Resten von Aminoglykosiden anbringen und somit eine Antibiotika Inaktivierung hervorrufen, als zwei Aminoglykosid-Resistenz Faktoren, die *M. abscessus* eine relevante, klinische Medikamentenresistenz verleihen; und iii) Bla_*Mab*, eine Breitspektrum-β-Laktamase der Klasse A, welche die meisten β-Laktam-Antibiotika effizient hydrolysiert, mit Ausnahme von Imipenem,

Meropenem und Cefoxitin, als den Hauptfaktor für die angeborene β-Laktam Resistenz in *M. abscessus*.

Diese Forschungsergebnisse erweitern die Grundlagenkenntnisse über mögliche Interventionen für den hoch resistenten Krankheitserreger *M. abscessus* und deuten auf die akute Notwendigkeit der Entwicklung neuer Substanzen hin, die den verschiedenen Resistenzmechanismen, vermittelt durch Arr_*Mab*, AAC(2'), Eis2 sowie Bla_*Mab*, wiederstehen können.

INTRODUCTION

1. The genus *Mycobacterium*

The genus *Mycobacterium* is the only member of the family *Mycobacteriaceae* and encompasses more than 150 species, including a number of medically important pathogens, such as *Mycobacterium tuberculosis*, that exact an alarming toll in human morbidity and mortality. The members of this genus are straight or slightly curved nonmotile rods, nonsporeformers, stain Gram-positive, acid-fast, and share an unusually high genomic DNA G+C content (62–70%). They have a cell wall rich in long chain fatty acid esters, known as mycolic acids, which renders the mycobacteria impervious to many aseptic solutions and antibiotics. The Greek prefix "myco" means "fungus", alluding to the mold-like appearance of mycobacterial colonies on the surface of solid media.[1]

Historically, mycobacteria have been classified into two major groups on the basis of their growth rate. Rapid growing mycobacteria (RGM) form colonies on selective media in less than 7 days, whereas slow growing mycobacteria (SGM) require greater time periods.[2] Generation times range from 2 hours for *Mycobacterium smegmatis* to 12 days for *Mycobacterium leprae*.[1] RGM species are usually harmless saprophytes, while SGM are frequently associated with mycobacterial diseases.[2] One remarkable exception to the concept of RGM as saprophytes is *Mycobacterium abscessus*, presently one of the most prevalent and the most drug-resistant opportunistic pathogen in human disease among RGM.[3]

2. Nontuberculous Mycobacteria (NTM)

For diagnostic and treatment purposes mycobacteria are further divided into three major groups: (i) *M. tuberculosis* complex, which comprises *M. tuberculosis* as well as other causative agents of animal and human tuberculosis, such as *Mycobacterium bovis* and *Mycobacterium africanum*, (ii) *Mycobacterium leprae*, which causes Hansen's disease or leprosy and (iii) nontuberculous mycobacteria (NTM), which encompass the rest of the mycobacterial species that do not cause tuberculosis or leprosy.[4] NTM are often environmental, ubiquitous organisms and are occasionally responsible for opportunistic infections throughout the human body with a broad spectrum of virulence.[5] The most common human infections attributed to NTM include pulmonary, skin and soft tissue infections and lymphadenitis.[6]

The incidence and prevalence of NTM pulmonary disease have been increasing worldwide and lately led to an emerging public health problem.[7] Despite the geographical distribution of NTM, by far, the most common organism associated with lung disease is the *Mycobacterium avium* complex (MAC), followed by *M. abscessus* complex and *Mycobacterium kansasii*.[8]

3. *Mycobacterium abscessus*

M. abscessus is the most pathogenic, chemotherapy-resistant and arduous to treat opportunistic pathogen among NTM and the most common isolated rapidly growing NTM associated with pulmonary disease.[9] Thus, *M. abscessus* has recently been described as an "antibiotic nightmare".[3]

3.1 History

M. abscessus was described for first time by Morris Moore and John Frerichs in 1953. These authors reported the isolation of a mycobacterium from a 62-year-old woman who had injured her left knee as a child when playing in a farm and had subcutaneous abscess-like lesions 48 years later. Due to the distinctive morphological, growing and biochemical characteristics of this mycobacterium, it was named as a new species, "*Mycobacterium abscessus*" (type strain *M. abscessus* ATCC 19977^T). The name of this new species "abscessus" was, hence, selected because of its ability to produce deep subcutaneous abscesses with a peripherally tuberculoid structure.[10]

3.2 Taxonomy

The taxonomic classification of *M. abscessus* remains presently in debate. After *M. abscessus* first description in 1953 and the recognition of *Mycobacterium chelonae* in 1972, these two RGM organisms were classified as two subspecies of the same species ("*M. chelonei*" or "*M. chelonae*").[11] Twenty years later, in 1992, *M. abscessus* was elevated to a species level and was separated by *M. chelonae*.[12]

After *M. abscessus* was recognized as an independent species, additional novel species *M. massiliense* and *M. bolletii*, closely related to *M. abscessus* (distinct *rpoB*), were

discovered.[13-14] Upon observation that *M. massiliense* and *M. bolletii* have a completely identical 16S rRNA sequence, Leao *et al*. proposed to combine *M. abscessus*, *M. massiliense* and *M. bolletii* in a single species, namely *M. abscessus*.[15] Within *M. abscessus* species, two subspecies particularly *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* were distinguished. The *M. abscessus* subsp. *bolletii* was proposed to include those isolates that were previously identified as *M. massiliense* or *M. bolletii*.[15]

Over the recent years, mounting evidence has emerged that the members of the *M. abscessus* subsp. *bolletii* designation are not homogenous.[16] While all of them are opportunistic pathogens in humans, they differ in susceptibility to macrolides due to sequence and functional diversity of their *erm* genes. In 2006, and while macrolides were the proposed treatment for most susceptible RGM species, Nash *et al*.[17] reported that the most clinically important RGM from the *Mycobacterium fortuitum* group (*Mycobacterium boenickei*, *Mycobacterium goodii*, *Mycobacterium houstonense*, *Mycobacterium mageritense*, *Mycobacterium neworleansense*, *Mycobacterium porcinum*, and *Mycobacterium wolinskyi*) possess an inducible erythromycin ribosomal methylase (*erm*) gene that confers macrolide resistance. Three years later, the same authors communicated the presence of a new *erm* gene, namely *erm(41)*, present in all taxa of *M. abscessus*.[18] However, the *erm(41)* gene of the former species *M. massiliense* has two deletions that render the gene non-functional and therefore, clinical isolates of *M. massiliense* do not show inducible resistance to macrolides, the primary therapeutic antibiotics for *M. abscessus*, and thereby, have more favorable treatment outcomes.[19]

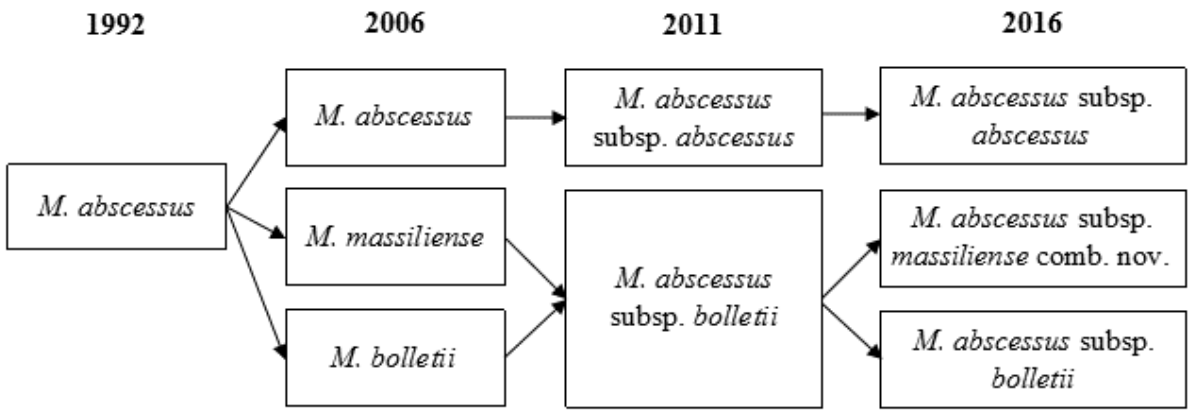

Figure 1. Serial nomenclatural and taxonomic changes of *Mycobacterium abscessus* complex, 1992-2016. Adapted from Lee MR, Sheng WH, Hung CC, *et al*. 2015.[4]

Recently, more light was shed on the taxonomic position of the members of *M. abscessus* complex by computational sequence analyses. According to prior *erm(41)*-related phenotypic data and genomic data from Tortoli *et al*., the authors concluded that the 3 members of *M. abscessus* complex do not meet the criteria for differentiation at the species level, but at the subspecies level. In that context, the authors suggest that *M. abscessus* comprises *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and a third subspecies for which the name *M. abscessus* subsp. *massiliense* comb. nov. was proposed. [20]

3.3 Etiopathogenesis

M. abscessus is an environmental opportunistic pathogen, found in soil, water and dust. It is able to survive in host cells, primarily in macrophages and fibroblasts and therefore is considered an intracellular pathogen.[21] "Smooth" (S) and "rough" (R) morphotypes of *M. abscessus* have been described, which are associated with less severe or more acute human infections, respectively.[22] Most clinically relevant cases are associated with pulmonary infections in patients with cystic fibrosis, bronchiectasis or pneumoconiosis and disseminated disease in immunocompromised individuals.[9,21-23] *M. abscessus* is highly resistant to disinfectants, as well, and thereby it often causes skin and soft tissue infections when nonsterile techniques are applied or contaminated materials are used during plastic surgery, tattooing, body piercing, mesotherapy and acupuncture procedures.[9,24-31] Several worldwide reported healthcare-associated outbreaks of *M. abscessus*, highlight the increasing medical importance of this multidrug-resistant pathogen and the urgent need for reliable medication strategies.[9,23,32]

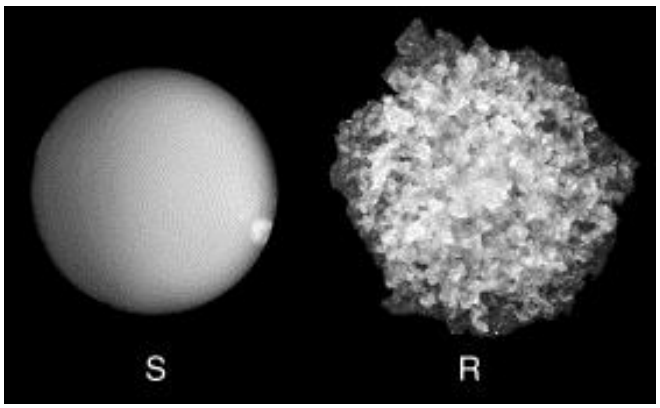

Figure 2. Smooth (S) and rough (R) morphotypes of *M. abscessus*. Figure from Medjahed H, Gaillard JL, Reyrat JM. 2010.[33]

3.4 Treatment

Treatment of an *M. abscessus* infection is long, costly and complicated due to the bacteria's high degree of innate resistance to antimicrobial agents.[34] Being intrinsically resistant towards most classes of antibiotics used for treatment of Gram-positive and Gram-negative bacterial infections, like β-lactams, aminoglycosides and macrolides, as well as towards the first-line antituberculosis drugs, *M. abscessus* has been recently characterized an "antibiotic nightmare".[3] Indeed, treatment options against *M. abscessus* infection are more limited than for *M. tuberculosis* infection.[3] While no standard treatment recommendations for *M. abscessus* infections have yet been established, antibiotic administration is empiric, should be individualized and therefore, heavily relies on *in vitro* antibiotic susceptibility testing (AST) by broth microdilution and definitive subspecies identification.[7,9]

The clinical importance of *M. abscessus* subspecies identification (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) is attributed to the fact that, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, have a functional inducible erythromycin ribosome methyltransferase [*erm(41)*] gene that confers macrolide resistance, whereas *M. abscessus* subsp. *massiliense* has a nonfunctional *erm(41)* leading to macrolide susceptibility and thereby, prognosis and treatment outcomes of *M. abscessus* subsp. *massiliense* infections are more propitious.[17-19,35]

Apt antibiotics are given for varying periods of time depending on severity and location of *M. abscessus* disease. Skin and soft tissue infections are often successfully treated within 3–6 months with excellent chance for cure when chemotherapy and concomitant surgical debridement are combined. Although there is no standard treatment for pulmonary *M. abscessus* infections, current guidelines from the American Thoracic Society propose periodic administration of a multidrug therapy, including an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to macrolides and the intravenous aminoglycoside amikacin in combination with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[36-38] However, treatment outcomes are extremely poor, often related to adverse effects and drug toxicities, while relapse rates are very high.[39] Lung disease due to *M. abscessus* is, therefore, only considered by clinicians "manageable", but not "curable".[40]

3.5 Antibiotic Susceptibility Testing (AST)

M. abscessus is notoriously resistant to standard antituberculosis drugs and most commonly administered commercial antibiotics. Therefore, treatment strategies followed by clinicians heavily rely on *in vitro* antibiotic susceptibility testing (AST) results.[3] The Clinical and Laboratory Standards Institute (CLSI), the only organization worldwide that has published AST guidelines for RGM, recommends susceptibility testing of amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline or minocycline, imipenem, linezolid, moxifloxacin, trimethoprim-sulfamethoxazole and tobramycin. The recommended method for *M. abscessus* AST is broth microdilution in cation-adjusted Mueller-Hinton broth.[41]

With the exception of the macrolide class and since clarithromycin became the cornerstone for oral *M. abscessus* treatment, very limited data concerning the correlation between AST and the clinical efficacy of the recommended antimycobacterial drugs have been reported in the literature.[42] Among the suggested chemotherapeutic agents for *M. abscessus*, clarithromycin, amikacin and cefoxitin have the best antimycobacterial activity.[9] However, the clinical relevance of AST remains controversial, because of technical problems associated with AST methods, reproducibility of AST results, significant discrepancies between *in vitro* susceptibility and *in vivo* activity of a given drug, solubility and stability issues of the drugs used.[43] For example, *M. abscessus* inducible macrolide resistance was for long unrecognized by Minimal Inhibitory Concentration (MIC) assays, as visual inspection of the MIC results was suggested after 3 days of incubation by CLSI.[44] Nowadays, it is known that additional incubation is needed to detect inducible macrolide resistance and MIC reading should be done after 14 days of incubation.[41]

Nevertheless, despite the problematic correlation of the AST results with the *in vivo* activity of recommended antimycobacterial agents and all the technical issues that logically question the clinical utility of AST, the latter still remains the golden standard that guides the therapeutic plans chosen by clinicians.[3,43]

3.6 Genomics of *M. abscessus*

The genome of *M. abscessus* subsp. *abscessus* (CIP 104536^{T}) consists of a 5,067,172-bp circular chromosome, that exhibits 93% of coding capacity (4920 predicted coding sequences)

and 64% of G+C content, and a 23-kb mercury resistance plasmid, which is almost identical to pMM23 from *Mycobacterium marinum*.[45]

The chromosome of *M. abscessus* subsp. *abscessus* encodes many virulence proteins and numerous enzymes which putatively modify and consequently inactivate antibiotics, thereby contributing to complications in treatment of this emerging pathogen.[3,45] After 2009, when the *M. abscessus* genome sequence became available[45], several research groups worldwide have been motivated to study *M. abscessus* antibiotic resistance mechanisms. At first, lack of tools for the genetic manipulation of *M. abscessus* severely delayed research endeavours.[3] Although progress to this direction has been recently done, (β-lactam and macrolide resistance determinants of *M. abscessus* have been discovered and experimentally confirmed)[46-47], generation of isogenic mutants by targeted gene deletion in *M. abscessus* is still considered challenging.

3.7 Genetic Markers

Implementation of essential and powerful tools for targeted gene deletion in *M. abscessus* would accelerate identification of *M. abscessus* drug resistance mechanisms. Very recently, significant progress has been made towards the development of conditional expression and homologous recombination systems for *M. abscessus*. However, these genetic strategies have not overcome the obstacles of low transformation efficiency, which typically leads to a limited number of mutants generated, and the high level spontaneous resistance to standard antibiotics used for selection, which gives rise to high background levels of false-positive colonies.[48-49] To date, the antibiotic resistance markers that have been applied for the generation of deletion mutants in *M. abscessus* include zeocin-, hygromycin-, kanamycin- and, only recently, apramycin resistance cassettes.[46-47,49-53]

Apramycin is a broad-spectrum atypical aminoglycoside antibiotic that has been applied in animal husbandry, but not in human disease treatment. Production of an aminoglycoside 3-*N*-acetyltransferase type IV [AAC(3)IV] confers cross-resistance to apramycin as well as a variety of other aminoglycosides. Mycobacterial species, such as *M. smegmatis* and *M. fortuitum* were known to be highly susceptible to apramycin compared with hygromycin and kanamycin.[54] Likewise, *M. tuberculosis* and *M. abscessus* are susceptible towards apramycin[55] and therefore the apramycin resistance gene has the potential to be used, and was recently used[53], as a marker gene for positive selection in *M. abscessus*.

Isoniazid, also known as isonicotinylhydrazide, is a first-line antituberculosis drug which targets InhA (enoyl-acyl carrier protein reductase), an enzyme involved in mycolic acid synthesis. As a pro-drug, isoniazid has to be activated by a catalase peroxidase, namely KatG. *M. abscessus* is assumed to be naturally resistant to isoniazid, because its pro-drug activator KatG is missing.[56] Heterologous expression of KatG, therefore, could serve as a negative selectable marker that could sensitize *M. abscessus* to isoniazid.

Once the barrier of lack of genetic tools for *M. abscessus* has been overcome, targeted deletion of genes putatively involved in drug resistance and virulence mechanisms of *M. abscessus* could be expedited.

3.8 Drug resistance of *M. abscessus*

Drug resistance of *M. abscessus* is conferred by a variety of mechanisms. Intrinsic resistance is attributed to low permeability of its complex and rich in lipids cell envelope, which acts as a physical and a chemical hydrophobic barrier. Furthermore, production of antibiotic-modifying/inactivating or drug-target-modifying enzymes and drug export systems contribute to intrinsic resistance. In *M. abscessus,* acquired drug resistance is usually conferred by mutations in the target genes, but eventually may also be conferred by horizontal gene transfer.[3,45] Since research attempts into *M. abscessus* drug resistance mechanisms are challenged by the limited number of genetic tools, most of the previous and up to date findings on *M. abscessus* drug resistance constitute hypotheses that were based on discoveries in other mycobacterial species, but could not be experimentally addressed in *M. abscessus*.[3]

3.8.1 Macrolide resistance

One major mechanism that renders *M. abscessus* resistant to chemotherapeutic agents is the modification of the drug target.[3] This mechanism has been extensively described in the context of *M. abscessus* macrolide resistance. Macrolide antibiotics (mainly azithromycin, clarithromycin, erythromycin and roxithromycin) are commonly used for treatment of non-tuberculous mycobacterial infections, including *M. abscessus*, *M. avium*, *Mycobacterium intracellulare* and *M. chelonae*.[3,9] Macrolides comprise a group of drugs whose activity stems from the presence of a macrolide ring, a large macrocyclic lactone ring to which one or more deoxy sugars may be attached. Macrolide antibiotics target the large ribosomal subunit,

preventing peptidyltransferase from adding the peptidyl group attached to tRNA to the next amino acid and inhibiting ribosomal translocation and thereby, protein synthesis.[57-58]

Nash *et al*. reported in 2006 for first time the presence of an inducible erythromycin ribosomal methylase (*erm*) gene, which confers macrolide resistance, in the genome of most clinically significant RGM species of the *M. fortuitum* group.[17] Bacterial macrolide resistance occurs by post-transcriptional methylation of the 23S rRNA at the *N*-6 position of adenosine 2058 (A2058) (*Escherichia coli* numbering), which is a pivotal nucleotide for the binding of macrolide antibiotics, thus inhibiting drug attachment.[59] Three years later (in 2009) and while macrolides were the treatment of choice for most susceptible RGM species, the same research group described a new *erm* gene in *M. abscessus* complex, designated as *erm(41)*.[18] *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* have an intact *erm* gene, which exhibits T/C polymorphism at the 28th nucleotide. On the one hand, the *erm(41)T28* gene product is induced by macrolides and methylates 23S rRNA, thus preventing macrolide binding to the ribosome. On the other hand, when cytidine is the 28th nucleotide of the *erm* gene [*erm(41)C28*], there is a loss of methylation function and thereby, macrolide resistance.[60] About 80% of *M. abscessus* subsp. *abscessus* clinical isolates and the vast majority of *M. abscessus* subsp. *bolletii* isolates have a wild-type *erm(41)* gene [*erm(41)T28*] and exhibit inducible macrolide resistance, characterized by low or intermediate MICs. The *erm(41)* gene of *M. abscessus* subsp. *massiliense* is shorter due to two deletions of nucleotides 64-65 and 159-432 which result in loss of gene function. As a consequence, such isolates of *M. abscessus* subsp. *massiliense* do not exhibit inducible macrolide resistance.[19,60-61] Finally, Choi *et al*. in 2012 experimentally confirmed, by using an *M. abscessus* subsp. *abscessus erm(41)*-knockout mutant and an *M. abscessus* subsp. *massiliense* transformant expressing *M. abscessus* subsp. *abscessus* wild-type *erm(41)*, that *erm(41)T28* is responsible for inducible *M. abscessus* macrolide resistance.[62]

Constitutive macrolide resistance, characterized by high MIC values, was described in all *M. abscessus* complex taxa as a consequence of a mutation at either the A2058 or the A2059 position of the 23S rRNA gene (*rrl*).[19,60-61] Generally, pathogenic species that develop macrolide resistance through mutations at A2058 (or neighbouring nucleotides) possess only one or two *rrn* operons. Indeed, *Mycobacterium* species possess only one *rrn* operon and therefore exhibit resistance to macrolides acquired by mutation in the *rrl* gene more frequent compared to other bacterial species.[63]

3.8.2 Rifamycin resistance

Rifampicin, from the group of rifamycin antibiotics, constitutes a semi synthetic product of the *Streptomyces* strain *Amycolatopsis rifamycinica*.[64] Although rifampicin is one of the most potent and broad spectrum antibiotics against bacterial infections and a key component of antituberculosis therapy[65], it shows only little activity towards *M. abscessus.* The bacterial activity of rifampicin stems from its binding with high affinity to the β subunit of the bacterial RNA polymerase thereby inhibiting transcription. Rifampicin binds in a pocket of the RNA polymerase β subunit deep in the DNA/RNA channel, but 12,1 Å away from the active site. Therefore, the RNA polymerase can bind and catalyze the formation of a phosphodiester bond between two substrate nucleotides in the presence of the antibiotic, but the antibiotic sterically blocks the path of the elongating RNA when the transcript becomes 2 to 3 nt long.[66-67]

Mutations within several "hotspots" in *rpoB*, the gene encoding the β subunit of RNA polymerase, is the most frequent mechanism of acquired rifampicin resistance among a variety of bacterial species, such as *E. coli*, *M. tuberculosis* and *Staphylococcus aureus*.[67-70] However, other resistance mechanisms have been also reported including limited membrane permeability to rifampicin and enzymatic inactivation of rifampicin.[71-76] Especially, *M. smegmatis* is naturally resistant to rifampicin, despite the lack of resistance associated polymorphisms in the *rpoB* target gene.[77] It is already proved that *M. smegmatis* produces an enzyme called rifampicin ADP-ribosyltransferase that adds an ADP-ribose group to rifampicin and thereby, hampers the drug's ability to bind to RNA polymerase.[74] The genome of *M. abscessus* encodes a putative rifampicin ADP-ribosyltransferase that is probably involved in *M. abscessus* high level of rifampicin resistance.[3]

3.8.3 Aminoglycoside resistance

Aminoglycosides are hydrophilic molecules consisting of a characteristic, central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bond(s). Aminoglycoside antibiotics primary target the bacterial ribosome and specifically, bind to the aminoacyl site of 16S ribosomal RNA within the 30S ribosomal subunit, leading to misreading of the genetic code and/or inhibition of translocation[78-80].

Aminoglycoside resistance typically occurs due to low uptake, energy-dependent bacterial efflux, target modification (16S rRNA methylation, mutation of the ribosomal target) or

enzymatic drug modification.[80] *M. abscessus* genome is supposed to code for numerous enzymes that are able to transfer acetyl, adenylyl and phosphate groups on crucial residues of aminoglycoside drugs leading to their inactivation. Particularly, *M. abscessus* encodes a putative aminoglycoside 2'-*N*-acetyltransferase and a number of homologues of additional aminoglycoside acetyltransferases and phosphotransferases.[45] It is hypothesized that these *M. abscessus* enzymes could confer aminoglycoside resistance, but so far no experimental evidence could confirm these speculations, which are primarily based on the fact that acetyltransferases and phosphotransferases from *M. smegmatis* and *M. tuberculosis* have been delineated to modify aminoglycoside drugs and endow these species with low level aminoglycoside resistance.[81] However, resistance levels of *M. abscessus* are much higher, eventually suggesting that some enzymes/mechanisms may act synergistically.[82]

3.8.4 β-lactam resistance

In addition to aminoglycoside resistance, *M. abscessus* also demonstrates high resistance to another important group of broad spectrum antibiotics, β-lactams. β-lactam antibiotics contain several subgroups of antibiotics, such as penicillins, monobactams, cephalosporins and carbapenems. All β-lactams contain a four-member β-lactam ring and selectively restrain the synthesis of the peptidoglycan layer of bacterial cell wall.[83-84] Genuinely, peptidoglycan is a polymer consisting of sugars and amino acids that forms a mesh-like layer outside the plasma membrane of bacteria, forming their cell wall. The sugar component consists of alternating residues of β-(1,4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid. Attached to the *N*-acetylmuramic acid is a peptide chain of three to five amino acids. The peptide chain is cross-linked to the peptide chain of another strand forming the 3-dimensional (3-D) mesh-like layer which provides the cell wall with strength. This 3-D network is basically established by transpeptidases or penicillin binding proteins (PBP).[85] β-lactams mimic D-alanyl-D-alanine, a component of the peptidoglycan, which is imperative for the formation of the crosslinks by the PBP. In that way, the transpeptidases are obstructed by β-lactams, no crosslinks are accordingly formed, cell wall lacks strength and is, hence, disrupted.[86]

M. abscessus encodes an Ambler class A β-lactamase (MAB_2875) which hydrolyses the β-lactam ring. This enzyme is insensitive to β-lactamase inhibitors (such as clavulanate, tazobactam and sulbactam) and closely resembles β-lactamases from Gram-negative bacteria

(*Pseudomonas luteola*, *Serratia fonticola*).[87] Most likely, MAB_2875 has been acquired by horizontal gene transfer.[45]

4. Aims of the study

M. abscessus possesses various genes, which are hypothesized to be involved in its high intrinsic rifamycin, aminoglycoside and β-lactam resistance.[34,45] However, the exact function, the molecular proof and the interplay of these genes remain under cover. Elucidation of drug resistance mechanisms heavily relies on the ability for genetic manipulation of *M. abscessus*, particularly on generation of mutants by targeted gene inactivation. However, research endeavours into *M. abscessus* are hampered by the limited number of available genetic tools.[46-53]

The aims of this Ph.D. Thesis were to:

- Establish selectable markers for the generation of mutants by targeted gene inactivation in *M. abscessus*.
- Decipher *M. abscessus* intrinsic rifamycin resistance mechanisms.
- Elucidate *M. abscessus* innate aminoglycoside resistance mechanisms.
- Characterize *M. abscessus* β-lactam resistance mechanisms.

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CHAPTER 1

Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591

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ABSTRACT

Objectives: Rifampicin, a potent first-line TB drug of the rifamycin group, shows only little activity against the emerging pathogen *Mycobacterium abscessus.* Reportedly, bacterial resistance to rifampicin is associated with polymorphisms in the target gene *rpoB* or the presence of enzymes which modify and thereby inactivate rifampicin. The aim of this study was to investigate the role of the *MAB_0591* (arr_{Mab})-encoded rifampicin ADP-ribosyltransferase (Arr_*Mab*) in innate high-level rifampicin resistance in *M. abscessus.*

Methods: Recombinant *Escherichia coli* and *Mycobacterium tuberculosis* strains expressing *MAB_0591* were generated, as was an *M. abscessus* deletion mutant deficient for *MAB_0591*. MIC assays were used to study susceptibility towards rifampicin and C25 carbamate-modified rifamycin derivatives.

Results: Heterologous expression of *MAB_0591* conferred rifampicin resistance to *E. coli* and *M. tuberculosis*. Rifamycin MIC values were consistently lower for the *M. abscessus* Δarr_{Mab} mutant as compared with the *M. abscessus* ATCC 19977 parental type strain. The rifamycin wild-type phenotype was restored after complementation of the *M. abscessus* Δarr_{Mab} mutant with arr_{Mab}. Further MIC data demonstrated that a C25 modification increases rifamycin activity in wild type *M. abscessus*. However, MIC studies in the *M. abscessus* Δarr_{Mab} mutant suggest that C25 modified rifamycins are still subject to modification by Arr_*Mab*.

Conclusions: Our findings identify Arr_*Mab* as the major innate rifamycin resistance determinant of *M. abscessus.* Our data also indicate that Arr_*Mab*-mediated rifamycin resistance in *M. abscessus* can only in part be overcome by C25 carbamate modification.

INTRODUCTION

Mycobacterium abscessus, an environmental saprophyte, is one of the most pathogenic and drug-resistant organisms among rapidly growing mycobacteria (RGM).[1-2] It accounts for ~80% of all lung infections due to RGM, particularly exacerbations of chronic lung disease in patients with cystic fibrosis or bronchiectasis. *M. abscessus* is also responsible for skin or soft tissue infections, usually following trauma, plastic surgery or esthetic procedures (tattooing and body piercing).[3-11] Currently, there is no reliable antibiotic regimen for the treatment of infections with *M. abscessus*, as this bacterium demonstrates a high level of intrinsic and acquired resistance to commonly administered antibiotics.[2-3,7,11]

Rifampicin is a major chemotherapeutic agent of the rifamycin group.[12-13] The rifampicin mechanism of action is based on its ability to inhibit transcription by binding with high affinity to the *rpoB*-encoded β subunit (RpoB) of the DNA-dependent RNA polymerase of prokaryotes.[14-15] Bacterial rifampicin resistance is mostly attributed to alterations in *rpoB*, responsible for decreased affinity of the RpoB for rifampicin.[15-18] Less frequent mechanisms of rifampicin resistance include limited membrane permeability to rifampicin and enzymatic inactivation of rifampicin through modification processes.[19-24]

Rifampicin resistance mutations have originally been identified and extensively studied in *Escherichia coli* and are mapped in four distinct sequence clusters within the *rpoB* gene, known as the N-terminal cluster (N) and clusters I, II and III.[25-30] It has subsequently been shown that the majority of the rifampicin resistance mutations occur within an 81 bp region of cluster I, denoted as the rifampicin resistance-determining region, across all bacterial species.[28,31-33] In *Mycobacterium tuberculosis*, high-level clinically acquired rifampicin resistance is almost always conferred by mutations in *rpoB*.[34] In contrast, *Mycobacterium smegmatis*, which is naturally resistant to rifampicin, has rifampicin ADP-ribosyltransferase activity conferring innate rifampicin resistance.[22,35-36] Combrink *et al*.[37] delineated that the mechanism of Arr_*Msm*-mediated rifampicin inactivation can be overcome by a series of 3-morpholino rifamycins in which the C25 acetate group of the rifamycin core was replaced by a bulky carbamate group. The C25 carbamate prevents ribosylation of the adjacent C23 alcohol catalyzed by Arr_*Msm* and thus C25 rifamycins exhibit improved antimicrobial activity compared with rifampicin against *M. smegmatis*.[37]

Whole genome sequencing revealed that *M. abscessus* carries a putative ADP-ribosyltransferase (Arr_*Mab*), encoded by *MAB_0591* (arr_{Mab}).[11,38] To date, no experimental

data have defined the exact function of *MAB_0591* nor confirmed its association with *M. abscessus* innate high-level rifampicin resistance.

We here used heterologous expression of *MAB_0591* and targeted deletion of *MAB_0591* in *M. abscessus* to identify Arr_*Mab* as the major relevant rifampicin resistance determinant in the type strain *M. abscessus* ATCC 19977. In addition, our finding that Arr_*Mab*-mediated rifampicin resistance in *M. abscessus* can hardly be overcome by rifamycin C25 carbamate modification has important consequences for the development of new rifamycin derivatives active against *M. abscessus.*

MATERIALS AND METHODS

RpoB amino acid sequence alignment

The RpoB sequences of the *E. coli* K-12, *M. tuberculosis* H37Rv, *M. smegmatis* mc^2155 and *M. abscessus* ATCC 19977 were collected from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein/). Multiple sequence alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Bacterial strains and growing conditions

(i) *E. coli* strains were cultivated in LB medium at 37 °C overnight. Ampicillin was added to the medium at a final concentration of 120 mg/L, when necessary. For all cloning steps, *E. coli* XL1-Blue MRF' or *E. coli* MC1061 were used, whereas *E.coli-gfp* control strain and *E.coli-*arr_{Mab} testing strain were used for Minimal Inhibitory Concentration (MIC) assays. (ii) *M. abscessus* strains were grown in Middlebrook 7H9 liquid medium or on LB agar plates at 37 °C for 5 days. When needed, apramycin was added at a final concentration of 50 mg/L. The *M. abscessus* ATCC 19977 type strain, *M. abscessus* Δarr_{Mab} mutant strain, *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain and *M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV* vector backbone control strain were used throughout this study. (iii) The *M. smegmatis* mc^2155 type strain was grown on LB agar plates at 37 °C for 3 days. (iv) *M. tuberculosis* strains were cultivated in Middlebrook 7H9 liquid medium or on Middlebrook 7H10 agar plates, at 37 °C for 2-4 weeks and apramycin was added at a final concentration of 50 mg/L,

when necessary. The *M. tuberculosis* H37Rv type strain, Mtb-*aac(3)IV* control strain and Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain were used in this study.

Antibiotics

Ampicillin, apramycin, amikacin, isoniazid, rifampicin, rifapentine and rifaximin were bought from Sigma-Aldrich, Switzerland. The following C25 modified rifamycin derivatives were synthesized according to the literature protocol:[37] (i) 5f: 25-O-Desacetyl-(4-methoxybenzylaminocarbonyl) 3-morpholino rifamycin S; (ii) 5k: 25-O-Desacetyl-{C-[3-(2-methoxy-phenyl)-isoxazol-5-yl]-methylaminocarbonyl} 3-morpholino rifamycin S; and (iii) 5l: 25-O-Desacetyl-[C-(3-pyridin-2-yl-isoxazol-5-yl)-methylaminocarbonyl] 3-morpholino rifamycin S. The identity of the compounds was confirmed by Nuclear Magnetic Resonance and Mass Spectrometry Analysis. Purity of each compound was >97% as analyzed by HPLC. Compounds were dissolved in H_2O or DMSO according to the manufacturer's recommendations, were filter sterilized, aliquoted into stock solutions of 5-50 g/L and finally stored at -20 °C.

Expression of *MAB_0591* in *E. coli*

MAB_0591 was amplified from *M. abscessus* ATCC 19977 genomic DNA by Phusion High-Fidelity DNA Polymerase PCR (5′-ATA TAT GCT CTT CTA GTA CGA TGC CCA ACT TTT TGA-3′ and 5′-TAT ATA GCT CTT CAT GCG TCA TAG ATG ACC GCG TTT CC-3′). Following initial cloning into the pINIT vector, the sequence-verified *MAB_0591* amplicon was inserted downstream of the arabinose inducible pBAD promoter into the multi-copy expression vector pBXNH3 via a fragment exchange (FX) cloning system.[39] The resulting *E. coli* MC1061 pBXNH3-*arr*$_{Mab}$ testing strain is referred to as *E.coli-arr*$_{Mab}$. As a control, *gfp* (green fluorescence protein) was amplified from pOLYG-*gfp-hyg* vector (5′-ATA TAT GCT CTT CTA GTA TCT CGA AGG GCG AGG AGC T-3′ and 5′-TAT ATA GCT CTT CAT GCC TTG TAC AGC TCG TCC ATG CCG-3′) and was cloned directly into pBXNH3 expression vector. The resulting *E. coli* MC1061 pBXNH3-*gfp* control strain expressing *gfp* is referred to as *E.coli-gfp*. Gene expression of *MAB_0591* and *gfp* was conducted by induction of 30 mL bacterial cultures (OD_{600} = 0.85-1) with L-arabinose at a final concentration of 0.2% v/v, at 37 °C for 4.5 h.

Expression of *MAB_0591* in *M. tuberculosis*

MAB_0591 (including its native promoter) was PCR amplified from *M. abscessus* ATCC 19977 genomic DNA using *Kpn*I-linker modified primers 5′-AG GGTACC CGG ATA TGT GCA GCG GCA TG-3′ and 5′-GA GGTACC CAC CGA AGC ACT GAA GGT GC-3′ and cloned into the *Kpn*I site of the pMV361-*aac(3)IV* vector to result in the pMV361-*aac(3)IV-MAB_0591* complementing vector. The control backbone vector [pMV361-*aac(3)IV*] and the complementing vector [pMV361-*aac(3)IV-MAB_0591*] were transformed in electrocompetent *M. tuberculosis* H37Rv type strain, as previously described for *Mycobacterium bovis* BCG.[40] In brief, 400 µL of *M. tuberculosis* H37Rv competent cells were mixed with 1 µg of supercoiled plasmid DNA and electroporated in a BioRad Gene pulser II (settings: 2.5 kV, 1000 Ohms and 25 µF). Following electroporation, cells were resuspended in 4 mL of 7H9-OADC-Tween and incubated for 20 h at 37 °C. Appropriate dilutions were plated on selective agar and after 3 weeks of incubation, single colonies were picked, restreaked and grown in liquid broth when necessary. The Mtb-*aac(3)IV* control strain and the Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain were obtained by positive selection on 7H10 plates containing apramycin. The presence of the *aac(3)IV* and/or the *MAB_0591* gene(s) in these strains was confirmed by colony PCR.

Deletion of *MAB_0591* in *M. abscessus*

An 1.3-kbp *Psc*I/*Not*I fragment from position 591427 to 592720 (5′ *arr*$_{Mab}$ flanking sequence) and an 1.3-kbp *Not*I/*Xba*I fragment from position 593015 to 594322 (3′ *arr*$_{Mab}$ flanking sequence) were PCR amplified using genomic DNA from *M. abscessus* ATCC 19977 (5′-GAAATT ACATGT GTC ACG ATC TCC TGG ACT GCC TC-3′, 5′-GAAA GCGGCCGC CAT GGA AGT ACG CAC CCG ATT CG-3′ and 5′-GATA GCGGCCGC CCG AAT TCA TGG AAA CCT TCC GGG-3′, 5′-G TCTAGA GTC CTG TGT GAA CAG GTC GGT G-3′, respectively) and stepwise cloned into the pSE-*katG-aac(3)IV* suicide vector resulting in the knock-out vector pSE-*katG-aac(3)IV-*Δ*MAB_0591*. Details on the construction of *M. abscessus* deletion mutant will be described elsewhere (A. Rominski, P. Selchow and P. Sander, unpublished results). Briefly, pSE-*katG-aac(3)IV-*Δ*MAB_0591* was transformed into electrocompetent *M. abscessus* ATCC 19977. For electroporation, 100 µL competent cells were mixed with 1-2 µg supercoiled plasmid DNA and electroporated in a BioRad Gene pulser II with the following settings: 2.5 kV, 1000 Ohms and 25 µF. After electroporation

cells were resuspended in 0.9 mL of 7H9 medium and incubated for 5 h with constant shaking (1000 rpm) at 37 °C. Appropriate dilutions were subsequently plated on selective agar and after 5 days of incubation, single colonies were picked, restreaked and grown in liquid broth when necessary. Transformants were selected on LB agar plates containing apramycin and identified by *aac(3)IV* PCR. Single crossover transformants were identified by Southern blot analysis with a 0.2-kbp *Eco*RI 5′ arr_{Mab} DNA probe (the same probe was also used for all subsequent Southern blot analyses) and subjected to counterselection on LB agar plates containing isoniazid (32 mg/L). Single colonies were screened for deletion of *MAB_0591* by PCR and the genotype was finally confirmed by Southern blot analysis. In this way, a 0.3-kbp region of the *MAB_0591* was deleted. For complementation of the *M. abscessus* Δarr_{Mab} mutant with *MAB_0591*, the previously described complementation vector pMV361-*aac(3)IV-MAB_0591* was transformed into the Δarr_{Mab} mutant strain. Transformation was verified by Southern blot analysis.

Susceptibility testing of *M. tuberculosis* strains

Drug susceptibility testing (DST) was performed using the MGIT 960 system as recommended by the manufacturer[41] and results were interpreted as described previously by Springer *et al*.[42] In brief, 0.5 mL dilutions of positive MGIT vials of the Mtb-*aac(3)IV* control strain and the Mtb-*aac(3)IV*-arr_{Mab} testing strain were inoculated in fresh vials containing constant levels of apramycin for plasmid maintenance and different concentrations of the test drugs.[43] Rifampicin was tested at concentrations of 1, 4 and 20 mg/L, while the control drug isoniazid was tested at concentrations of 0.1, 1, 3 and 20 mg/L, respectively. For the drug free growth control, the bacterial working suspension was diluted 1:100 with sterile NaCl and 0.5 mL of the diluted working suspension was inoculated into the tube (proportion testing).[44] All MGIT tubes were incubated in the MGIT 960 instrument and monitored using the EpiCenter (version 5.53) software equipped with the TB eXiST module (Becton Dickinson, Switzerland).

Rifampicin Etest

Bacterial suspensions of *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab} and *M. abscessus* Δarr_{Mab}-arr_{Mab} strains were adjusted to a McFarland standard of 0.50 and were subsequently spread on LB agar plates using a sterile cotton swab. Then, a rifampicin Etest strip

(Biomérieux, Switzerland) was placed on each plate and plates were incubated for 5 days at 37 °C. The point of intersection between bacterial growth and the Etest device was read as MIC value.

MIC assays

MIC assays were performed according to the CLSI guidelines.[45] Working solutions were prepared by diluting the antibiotic stock solutions in Cation-Adjusted Müller-Hinton Broth (CAMHB; pH 7.4) (Becton Dickinson, Switzerland) to a concentration corresponding to twice the desired final concentration [working solutions of 128, 512 and 1024 mg/L were prepared, when the highest concentrations tested at the MIC assay were 64, 256 and 512 mg/L, respectively (Table 1, Table 3)]. By using CAMHB in sterile 96-well microtiter plates (Greiner Bio-One, Switzerland), two-fold serial dilutions of the working solutions were prepared. A positive growth control lacking antibiotic and a sterile negative control containing only CAMHB were included in each 96-well microtiter plate. For the preparation of the inoculum, 3-4 colonies from each bacterial strain grown on LB agar were transferred into a glass tube containing 2 mL of NaCl using a sterile cotton swab. In order to achieve a final inoculum titer of 1-5 x 10^5 cfu/mL, all bacterial suspensions were adjusted to a McFarland standard of 0.50 and subsequently diluted in CAMHB. The final test volume in each well of the microtiter plate was 0.1 mL. The correct titer of each inoculum was checked by obtaining cfu counts on LB agar plates. All microdilution plates were capped with adhesive sealing covers and incubated at 37 °C for i) 16 h for *E. coli* strains and ii) 3, 5, 7 and 12 days for *M. abscessus* and *M. smegmatis* strains, before the MIC values were assessed by visual inspection. All MIC assays were conducted in triplicate.

RESULTS AND DISCUSSION

Analysis of the RpoB rifampicin resistance sequence clusters from *M. abscessus*

To investigate whether innate rifampicin resistance in *M. abscessus* (MIC: 128 mg/L) is associated with polymorphisms in the RNA polymerase β-subunit, we aligned the RpoB amino acid sequence of *M. abscessus* ATCC 19977 with those of *E. coli* K-12 and *M. tuberculosis* H37Rv, known to be naturally susceptible to rifampicin,[13,16] and *M. smegmatis* mc^2155 that has no polymorphism in its *rpoB* gene corresponding to any known rifampicin

resistance genotype,[35] but is naturally resistant to rifampicin through ADP-ribosylation of rifampicin.[22,36] Our analysis revealed no polymorphism known to confer rifampicin resistance within all four (N, I, II and III) rifampicin resistance sequence clusters of *M. abscessus* RpoB (Figure 1), suggesting that other mechanisms are involved in the high intrinsic rifampicin resistance of *M. abscessus*. Possible mechanisms include efflux of rifampicin, diminished uptake of rifampicin, and enzymatic degradation or modification of rifampicin.[19-23] The latter resistance mechanism has been studied in *M. smegmatis* and in *Legionella pneumophila*.[22-23] A recent genome analysis suggested that *M. abscessus* carries a putative rifampicin ADP-ribosyltransferase (Arr_*Mab*),[38] encoded by *MAB_0591* (arr_{Mab}). MAB_0591 has 66% amino acid sequence identity with the *M. smegmatis* ADP-ribosyltransferase that modifies rifampicin. However, Table S1, which lists the MIC values of selected mycobacterial standard strains[42,46-47] as well as information about the presence or absence of ADP-ribosyltransferase-like protein in these species, shows that a direct correlation between rifampicin MIC and the presence or absence of ADP-ribosyltransferase-like proteins does not exist. *M. tuberculosis* and *M. leprae* were predicted to have no ADP-ribosyltransferase-like proteins and are susceptible to rifampicin (MIC: <1 mg/L).[42,47] ADP-ribosyltransferases were predicted to be present in *M. abscessus* (*M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*) and *M. fortuitum* and these species are resistant (MIC: ≥64 mg/L).[46] In contrast, the presence of putative ADP-ribosyltransferases in e.g. *Mycobacterium phlei*, *Mycobacterium gilvum* and *Mycobacterium marinum* does not correlate with high rifampicin MIC levels (MIC: <0.5 mg/L).[46] Therefore a functional role of ADP-ribosyltransferases in rifampicin resistance has to be addressed experimentally. By using ADP-ribosyltransferase of *M. smegmatis* mc^2155 in a BLASTP search, we identified also other, non-mycobacterial species predicted to possess ADP-ribosyltransferase-like proteins (Table S2). Interestingly, mycobacterial ADP-ribosyltransferases show homology to the catalytic domain of Exotoxin A from *Pseudomonas aeruginosa*,[48-49] pointing to a putative role of mycobacterial ADP-ribosyltransferases as virulence factors. However, within this study we exclusively focus on the role of *MAB_0591* in rifamycin resistance.

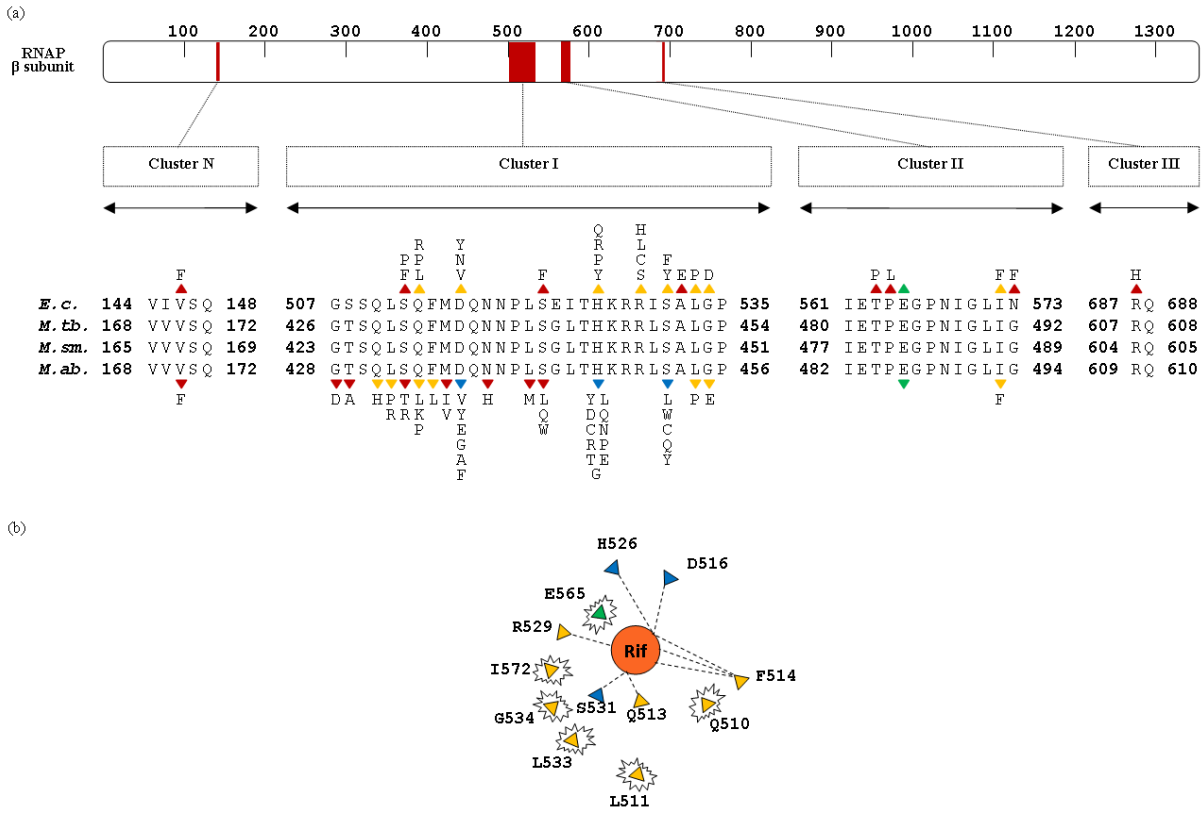

Figure 1. Rifampicin resistance regions of RpoB and their association with the interactions between rifampicin and the prokaryotic RNA polymerase (RNAP). (a) The bar on top illustrates the RNAP β subunit from *E. coli*. Directly above its amino acid numbering is indicated. Dark red lines within the bar indicate the position of the four clusters [N-terminal (N), and clusters I, II and III (I, II and III)] where the rifampicin resistance conferring alterations are identified across all bacterial species.[24-30] Directly below follows the amino acid sequence alignment spanning the rifampicin resistance regions of the *E. coli* K-12, *M. tuberculosis* H37Rv, *M. smegmatis* mc^{2}155 and *M. abscessus* ATCC 19977 RpoB. Amino acid substitutions that confer rifampicin resistance are shown as coloured triangles above and below the alignment, respectively. The possible substitutions for each position are mentioned in single amino acid code in columns directly above (for *E. coli*) or below the coloured triangles (for *M. tuberculosis*). The yellow triangles represent the residues that interact directly with rifampicin binding. In contrast, the red triangles indicate residues that are positioned too far away to have direct interaction with rifampicin. The blue triangles show the positions that are substituted with the highest frequency among *M. tuberculosis* rifampicin-resistant isolates.[31] The green triangles show a residue that is directly interacting with rifampicin, but no substitutions have been reported at this position, probably because they would be fatal for the bacterium.[15] (b) Using the same colour code for the amino acid positions as above (a), the residues of direct interaction with rifampicin (Rif) are shown (yellow, blue and green triangles). Hydrogen bonds formed between an RpoB residue and rifampicin are depicted as dashed lines and residues that form van der Waals interactions are indicated in zigzag circles. Numbering of the residues is according to the *E. coli* positions.[15]

Heterologous expression of *MAB_0591* in *E. coli* and *M. tuberculosis*

To study if rifampicin resistance in *M. abscessus* is due to arr_{Mab}, we cloned *MAB_0591* and expressed the recombinant protein in two rifampicin susceptible hosts, *E. coli*[16] and *M. tuberculosis*.[50] First, we addressed the question whether induced expression of *MAB_0591* would confer rifampicin resistance in a non-mycobacterial heterologous host. Arr_*Mab* was recombinantly expressed by the multi-copy pBXNH3-arr_{Mab} vector in *E. coli* MC1061 under the control of the L-arabinose inducible pBAD promoter. The *E.coli-gfp* control strain and the *E.coli-*arr_{Mab} testing strain were generated following transformation with the pBXNH3-*gfp* and the pBXNH3-arr_{Mab} vectors, respectively. The recombinant strains were subsequently tested against rifampicin in MIC assays. The *E.coli-*arr_{Mab} testing strain was highly resistant to rifampicin with MIC values >512 mg/L, while the *E.coli-gfp* control strain remained susceptible to rifampicin (MIC: 4 mg/L) (Table 1). These results confirm that Arr_*Mab* plays a critical role as a rifampicin resistance determinant when expressed in the rifampicin susceptible *E. coli* host. We hypothesize that Arr_*Mab* modifies rifampicin by ADP-ribosylation at the hydroxyl group of C23, as previously shown for Arr_*Msm*[48] and that this modification interferes with target binding.

Table 1. DST results of *E. coli* expressing *MAB_0591*[*]

Strains	Rifampicin MIC (mg/L)
E.coli-gfp	4
E.coli-arr_{Mab}	>512

[*]Broth microdilution method

Next, we studied whether arr_{Mab} confers rifampicin resistance in a mycobacterial host, *M. tuberculosis*. In order to simulate veritable levels of Arr_*Mab* expression, we expressed MAB_0591 under control of its native promoter. The single-copy integrating plasmids pMV361-*aac(3)IV* and pMV361-*aac(3)IV-MAB_0591* were engineered and transformed into the rifampicin susceptible *M. tuberculosis* H37Rv strain (MIC: <1 mg/L).[42] The *aac(3)IV* gene that confers resistance towards apramycin was used as positive selection marker. The resulting strains referred to as Mtb-*aac(3)IV* control strain and Mtb-*aac(3)IV-*arr_{Mab} testing strain respectively, were subjected to DST using the MGIT 960 system equipped with the EpiCenter TB eXiST software. Susceptibility to different concentrations of rifampicin (1, 4

and 20 mg/L) and the non-substrate control drug isoniazid (0.1, 1, 3 and 10 mg/L) was determined. The Mtb-*aac(3)IV* control strain was, as expected, susceptible to both rifampicin and isoniazid at all concentrations tested (MIC: rifampicin <1.0 mg/L; isoniazid <0.1 mg/L). In contrast, the Mtb-*aac(3)IV-arr*$_{Mab}$ testing strain was specifically resistant to rifampicin, even at the highest concentration tested (MIC: >20 mg/L), but remained susceptible to the unrelated control drug isoniazid (MIC: <0.1 mg/L) (Table 2). These findings demonstrate that *MAB_0591* confers high-level resistance to rifampicin, but not to other drugs, when expressed in a rifampicin-susceptible mycobacterial host.

Table 2. DST results of *M. tuberculosis* expressing *MAB_0591*[**]

	Rifampicin			Isoniazid
Strains	1.0 mg/L	4.0 mg/L	20.0 mg/L	0.1 mg/L
Mtb-*aac(3)IV*	S	S	S	S
Mtb-*aac(3)IV-arr*$_{Mab}$	R	R	R	S

[**]Proportion method using the MGIT 960 system

S=susceptible; R=resistant

Generation of *M. abscessus MAB_0591* deletion mutant

Heterologous expression of *MAB_0591* indicated that *arr*$_{Mab}$ is able to confer rifampicin resistance to a susceptible host; however its role in innate rifampicin resistance in *M. abscessus* remained to be determined. We recently developed tools for genetic manipulation of *M. abscessus* (A. Rominski, P. Selchow and P. Sander, unpublished results) and wished to exploit this technique to generate an *M. abscessus MAB_0591* deletion mutant. This mutant would allow us to directly address the role of MAB_0591 in innate rifampicin resistance. The *arr*$_{Mab}$ deletion mutant was constructed by transformation of *M. abscessus* ATCC 19977 with suicide plasmid pSE-*katG-aac(3)IV-*Δ*MAB_0591* applying apramycin positive selection[51] and a *katG*-dependent isoniazid counterselection strategy that we previously established (A. Rominski, P. Selchow and P. Sander, unpublished results) (Figure 2a). Deletion of *MAB_0591* was confirmed by Southern blot analysis (Figure 2b). A complemented mutant strain was constructed by transformation of *M. abscessus* Δ*arr*$_{Mab}$ mutant with the complementation vector pMV361-*aac(3)IV-MAB_0591* expressing Arr_*Mab*. The complemented mutant strain

is referred to as *M. abscessus* Δarr_{Mab}-arr_{Mab}. Genetic complementation was confirmed by Southern blot analysis (Figure 2b).

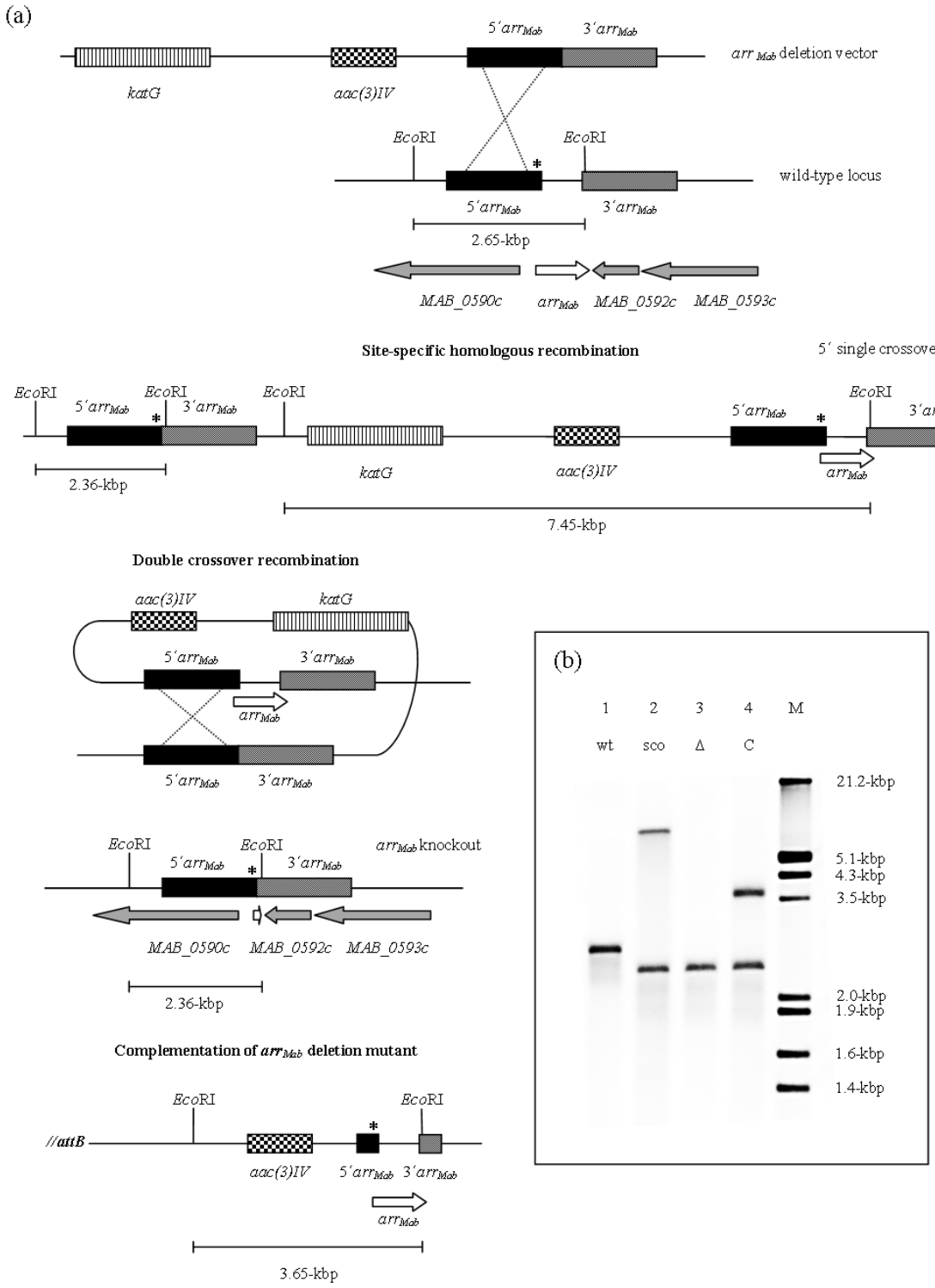

Figure 2. Genotypic analyses of *M. abscessus* arr_{Mab} locus. (a) Schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirms the deletion of *MAB_0591* from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1), *M. abscessus* transformant with arr_{Mab} targeting vector [pSE-*katG-aac(3)IV-*Δ*MAB_0591*] prior to (2) and after KatG-dependent isoniazid-counterselection (3)

and after transformation of counterselected mutant with arr_{Mab} complementation vector (4) was digested with *Eco*RI and probed with a fragment from the 5′ arr_{Mab} flanking region. M = Molecular Marker. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 2.65-kbp fragment of the wild-type (wt) parental strain, to the 2.36-kbp and 7.45-kbp fragments after site-specific homologous recombination [single crossover (sco)], to a 2.36-kbp fragment of the Δarr_{Mab} mutant (Δ) and to the 2.36-kbp and 3.65-kbp fragments of the *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain (C).

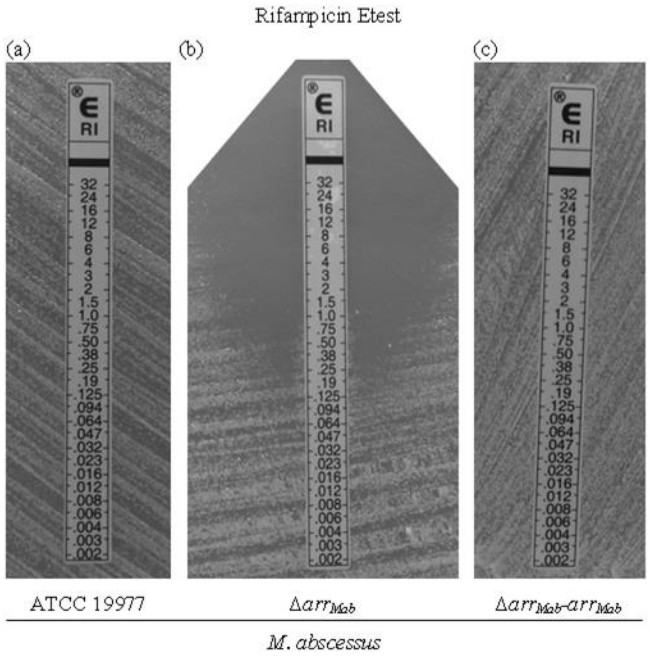

Figure 3. Contribution of *MAB_0591* to rifampicin resistance in *M. abscessus.* Rifampicin susceptibility of (a) *M. abscessus* ATCC 19977 wild-type strain, (b) *M. abscessus* Δarr_{Mab} mutant and (c) *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant strain, was examined by Etest. The picture of the rifampicin Etest results was taken after 5 days of incubation at 37 °C.

DST of *M. abscessus* Δ*MAB_0591*

For determination of the Δarr_{Mab} mutant's phenotype, a rifampicin Etest was carried out with *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab} mutant and *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented strain. Etest results after 5 days of incubation at 37 °C revealed high-level rifampicin resistance in *M. abscessus* ATCC 19977 (MIC: >32mg/L). In contrast, the Δarr_{Mab} mutant showed susceptibility to low rifampicin concentrations (MIC: ≈0.5 mg/L). Rifampicin resistance was restored upon complementation of the Δarr_{Mab} mutant strain with arr_{Mab} [(MIC: >32 mg/L); Figure 3]. MIC values were subsequently determined in detail for rifampicin, rifapentine and rifaximin. *M. abscessus* ATCC 19977, *M. abscessus* Δarr_{Mab}, *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant, *M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV*

vector backbone control strain and *M. smegmatis* mc^2155 were subjected to DST. For *M. abscessus* Δarr_{Mab} the MICs of all rifamycins were consistently and significantly lower than for *M. abscessus* ATCC 19977 wild-type strain, indicating that all tested rifamycins are modified by Arr_*Mab* (Table 3). Transformation of *M. abscessus* Δarr_{Mab} mutant with $arr_{Mab,}$ restored wild-type levels of rifamycin resistance while transformation with the empty vector backbone did not. Expectedly, amikacin MICs were independent of the *arr* genotype. These findings identify Arr_*Mab* as the major rifamycin resistance determinant in *M. abscessus*.

DST of C25 modified rifamycin derivatives

Carbamate modification at the C25 position of the rifamycin core has been shown to improve antimicrobial activity against *M. smegmatis* mc^2155.[37] We wanted to test whether these compounds also overcome rifamycin resistance in *M. abscessus*. C25 modified rifamycin derivatives 5f, 5k and 5l (Figure 4) were custom synthesized and tested for antimicrobial activity. The compounds showed potent activity against *M. smegmatis* – on average these compounds were 100 – 200-fold more active than rifampicin. These results confirm former findings that C25 modified rifamycins apparently are resilient to modification by Arr_*Msm*. The C25 modified rifamycins also showed increased activity in *M. abscessus* as compared to rifampicin, rifapentine and rifaximin (Table 3). Of note, C25 modification not only increases rifamycin activity against the *M. abscessus* wild type, but also against the *M. abscessus* Δarr_{Mab} mutant, although to a lesser extent. These data indicate that the increased activity of C25 rifamycin derivatives is only partially due to resilience towards Arr_*Mab*-modification. Compared to compounds 5f and 5k, we observed little time-dependent increase of the MIC values for compound 5l in wild type *M. abscessus* and *M. abscessus* Δarr_{Mab}-arr_{Mab} complemented mutant, indicating that 5l is probably least modified by Arr_*Mab*, but still wild-type MIC values are high (4 mg/L; 16-fold higher than against the Δarr_{Mab} mutant).

Table 3. DST results of *M. abscessus* and *M. smegmatis* strains*

Strains	*M. abscessus* ATCC 19977				*M. abscessus* Δarr_{Mab}				*M. abscessus* Δarr_{Mab}-arr_{Mab}				*M. abscessus* Δarr_{Mab} pMV361-*aac(3)IV*				*M. smegmatis* mc^{2}155			
Day	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12
Antibiotic	MIC (mg/L)																			
Rifampicin	128	256	256	>256	0.25	0.5	1	1	128	256	256	>256	0.25	0.5	1	1	128	128	128	128
Rifapentine	256	>256	>256	>256	1	2	2	4	128	256	>256	>256	1	2	2	4	64	128	128	128
Rifaximin	64	128	256	256	1	2	4	8	64	128	256	>256	1	2	4	4	256	256	>256	>256
5f	8	>64	>64	>64	0.016	0.063	0.063	0.25	8	64	>64	>64	0.016	0.125	0.125	0.25	0.125	0.25	0.25	0.5
5k	4	32	>64	>64	0.063	0.125	0.25	0.25	4	16	>64	>64	0.031	0.125	0.25	0.25	0.25	0.25	1	1
5l	2	4	4	8	0.0078	0.125	0.25	0.25	2	4	4	4	0.016	0.125	0.25	0.25	0.25	0.5	1	1
Amikacin	1	2	4	4	1	2	4	4	1	2	4	4	1	2	4	4	<0.5	<0.5	<0.5	<0.5

* Broth microdilution method

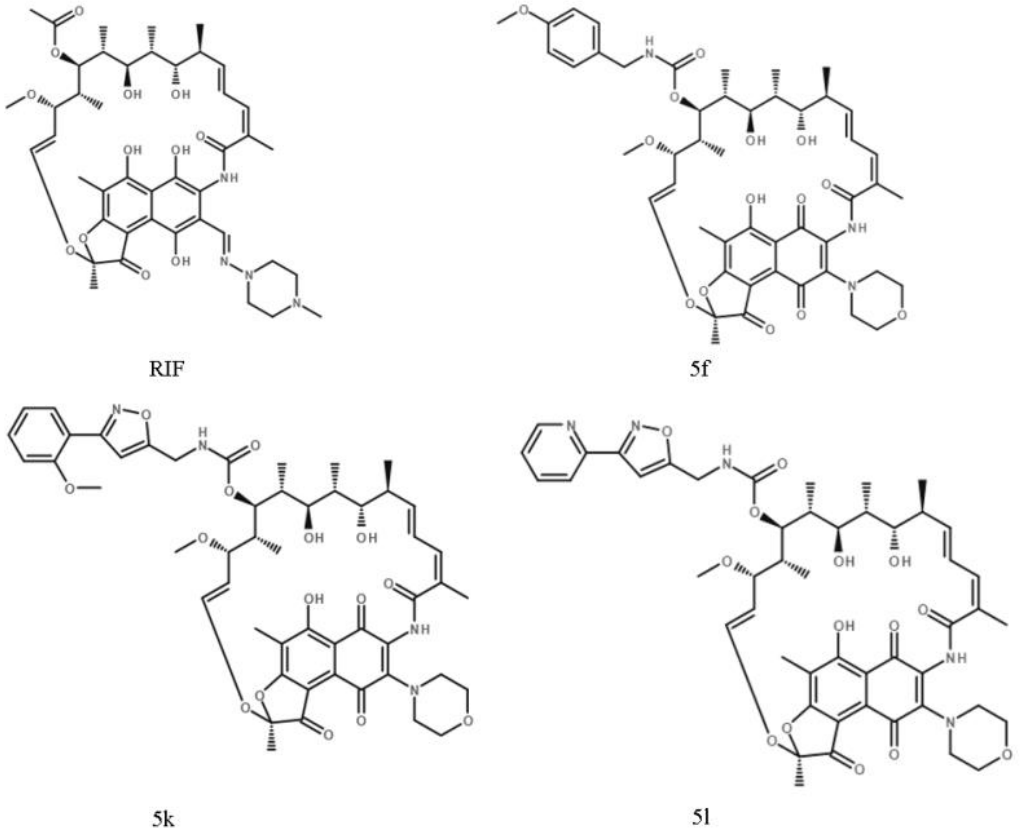

Figure 4. Rifampicin (RIF) and the 5f: 25-O-Desacetyl-(4-methoxybenzylaminocarbonyl) 3-morpholino rifamycin S, 5k: 25-O-Desacetyl-{C-[3-(2-methoxy-phenyl)-isoxazol-5-yl]-methylaminocarbonyl} 3-morpholino rifamycin S and 5l: 25-O-Desacetyl-[C-(3-pyridin-2-yl-isoxazol-5-yl)-methylaminocarbonyl] 3-morpholino rifamycin S, C25 carbamate rifamycin derivatives, which are numbered per the original work,[37] for ease of comparison.

Conclusions

Taken together, our study identified Arr_*Mab* as the major determinant of innate rifamycin resistance in *M. abscessus.* Our data indicate significant species-specific differences in rifamycin-C25-mediated resilience for Arr_*Msm* and Arr_*Mab*, since Arr_*Mab*-mediated rifamycin resistance can only partly be overcome by C25 modification. These findings testify to the need to develop novel compounds that are able to escape Arr_*Mab*-mediated rifamycin resistance in *M. abscessus*. Structural similarity between Arr_*Msm* and protein ADP-ribosyltransferases, prominently domain III of *Pseudomonas aeruginosa* exotoxin A have been described.[48-49] These structural features are also conserved in Arr_*Mab*. Therefore, it is tempting to speculate on a dual role of Arr_*Mab* in drug resistance and virulence.

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SUPPLEMENTARY DATA

Table S1. Distribution of ADP-ribosyltransferase-like proteins in selected mycobacterial species and rifampicin MIC

Species (Standard Strain)	**RGM/SGM**	**ADP-ribosyltransferase**	**MIC rifampicin (mg/L)[42,46-47]**
M. smegmatis mc^2155	RGM	+	128
M. abscessus subsp. *abscessus* (ATCC 19977)	RGM	+	>256
M. abscessus subsp. *bolletii* (CIP 108541)	RGM	+	>256
M. tuberculosis H37Rv	SGM	-	<1
M. leprae Thai-53	SGM	-	<1
M. chelonae (NCTC 946)	RGM	+	64
M. fortuitum (ATCC 6841)	RGM	+	64
M. peregrinum (ATCC 14467)	RGM	+	2
M. doricum (ATCCBAA 565)	RGM	-	64
M. obuense (ATCC 27023)	RGM	+	16
M. phlei (ATCC 11758)	RGM	+	<0.5
M. duvalii (ATCC 43910)	RGM	-	<0.5
M. parafortuitum (ATCC 19686)	RGM	-	4
M. gilvum (ATCC 43909)	RGM	+	<0.5
M. flavescens (ATCC 14474)	RGM	-	16
M. intracellulare (ATCC 13950)	SGM	-	2
M. xenopi (NCTC 10042)	SGM	+	1
M. senegalense (ATCC 35796)	SGM	-	2
M. gordonae (ATCC 14470)	SGM	-	<0.5
M. marinum (ATCC 927)	SGM	+	<0.5
M. kansasii (ATCC 12478)	SGM	-	<0.5
M. scrofulaceum (ATCC 19981)	SGM	+	0.5
M. malmoense (ATCC 29571)	SGM	-	1
M. avium (ATCC 25291)	SGM	-	8
M. szulgai (NCTC 10831)	SGM	+	<0.5
M. terrae (ATCC 15755)	SGM	-	1
M. simiae (ATCC 25275)	SGM	+	8

RGM= rapidly growing mycobacteria; SGM= slow growing mycobacteria

Table S2. Distribution of ADP-ribosyltransferase-like proteins in non-mycobacterial species

Description	Query coverage	E-value	Identity	Accession
NAD(+)-rifampin ADP-ribosyltransferase [*Rhodococcus opacus*]	100%	1.00E-77	79%	WP_043826678.1
NAD(+)-rifampin ADP-ribosyltransferase [*Gordonia bronchialis*]	98%	5.00E-76	79%	WP_012832191.1
rifampin ADP-ribosyl transferase [*Cryobacterium arcticum*]	100%	1.00E-73	76%	WP_066598497.1
rifampin ADP-ribosyl transferase [*Gordonia polyisoprenivorans*]	100%	1.00E-70	73%	WP_006368867.1
rifampin ADP-ribosyl transferase [*Cellulomonas fimi*]	100%	1.00E-68	70%	WP_013771728.1
rifampin ADP-ribosyl transferase [*Cryobacterium roopkundense*]	100%	6.00E-68	70%	WP_035838358.1
rifampin ADP-ribosyl transferase [*Ornithinimicrobium pekingense*]	97%	5.00E-67	70%	WP_022921799.1
rifampin ADP-ribosyl transferase [*Microlunatus phosphovorus*]	97%	6.00E-67	70%	WP_041790446.1
rifampin ADP-ribosyl transferase [*Williamsia herbipolensis*]	100%	4.00E-66	70%	WP_045824634.1
rifampin ADP-ribosyl transferase [*Streptomyces flavochromogenes*]	100%	4.00E-66	66%	WP_030319906.1
rifampin ADP-ribosyl transferase [*Serinicoccus profundi*]	97%	1.00E-65	70%	WP_010146955.1
rifampin ADP-ribosyl transferase [*Streptomyces durhamensis*]	100%	1.00E-64	67%	WP_031173901.1
rifampin ADP-ribosyl transferase [*Propionicicella superfundia*]	97%	5.00E-63	70%	WP_028709133.1
rifampin ADP-ribosyl transferase [*Lysinimicrobium gelatinilyticum*]	99%	4.00E-62	68%	WP_062516799.1
rifampin ADP-ribosyl transferase [*Nocardiopsis kunsanensis*]	100%	7.00E-62	67%	WP_017576663.1
rifampin ADP-ribosyl transferase [*Arthrobacter alpinus*]	97%	3.00E-61	68%	WP_062289126.1
rifampin ADP-ribosyl transferase [*Cellulomonas flavigena*]	100%	5.00E-61	65%	WP_013115596.1
rifampin ADP-ribosyl transferase [*Rhodococcus fascians*]	100%	6.00E-61	64%	WP_032395579.1
rifampin ADP-ribosyl transferase [*Demequina globuliformis*]	100%	1.00E-60	67%	WP_062076570.1
rifampin ADP-ribosyl transferase [*Gordonia terrae*]	100%	4.00E-60	62%	WP_033206549.1
rifampin ADP-ribosyl transferase [*Williamsia muralis*]	100%	8.00E-60	62%	WP_062794673.1
rifampin ADP-ribosyl transferase [*Aeromicrobium marinum*]	100%	1.00E-59	64%	WP_007077209.1
rifampin ADP-ribosyl transferase [*Lysinimicrobium rhizosphaerae*]	100%	2.00E-59	66%	WP_062526649.1
rifampin ADP-ribosyl transferase [*Lysinimicrobium subtropicum*]	97%	6.00E-59	68%	WP_062301052.1
rifampin ADP-ribosyl transferase	100%	7.00E-59	66%	WP_062318234.1

[*Lysinimicrobium aestuarii*]				
rifampin ADP-ribosyl transferase [*Streptomyces lavenduligriseus*]	100%	6.00E-58	68%	WP_030788496.1
rifampin ADP-ribosyl transferase [*Actinotalea ferrariae*]	100%	1.00E-57	63%	WP_034227635.1
rifampin ADP-ribosyl transferase [*Gordonia namibiensis*]	100%	2.00E-57	60%	WP_040535037.1
rifampin ADP-ribosyl transferase [*Streptomyces globisporus*]	100%	2.00E-56	65%	WP_030691134.1
rifampin ADP-ribosyl transferase [*Gordonia amicalis*]	100%	3.00E-56	60%	WP_024497540.1
rifampin ADP-ribosyl transferase [*Gordonia kroppenstedtii*]	97%	5.00E-56	70%	WP_026303538.1
rifampin ADP-ribosyl transferase [*Streptomyces venezuelae*]	100%	3.00E-55	65%	WP_055639481.1
rifampin ADP-ribosyl transferase [*Nocardia farcinica*]	93%	4.00E-55	61%	WP_068971096.1
rifampin ADP-ribosyl transferase [*Gordonia paraffinivorans*]	96%	7.00E-55	63%	WP_006900611.1
hypothetical protein [*Cellulomonas massiliensis*]	95%	1.00E-54	63%	WP_019136146.1
rifampin ADP-ribosyl transferase [*Flavihumibacter petaseus*]	88%	4.00E-53	63%	WP_046368559.1
rifampin ADP-ribosylating transferase ARR-2 [*Modestobacter marinus*]	92%	7.00E-53	63%	WP_051144103.1
rifampin ADP-ribosylating transferase [*Modestobacter marinus*]	92%	1.00E-52	63%	CCH85944.1
rifampin ADP-ribosyl transferase [*Clostridium propionicum*]	92%	4.00E-52	63%	WP_066053732.1
rifampin ADP-ribosylating transferase [*Saccharomonospora paurometabolica*]	88%	2.00E-51	63%	WP_007026413.1
rifampin ADP-ribosyl transferase [*Pontibacter actiniarum*]	98%	2.00E-51	56%	WP_025607027.1
rifampin ADP-ribosylating transferase [*Sphingobacterium spiritivorum*]	89%	2.00E-51	60%	WP_003006667.1
rifampin ADP-ribosyl transferase [*Desulfitobacterium hafniense*]	96%	5.00E-51	58%	WP_005811445.1
rifampin ADP-ribosyl transferase [*Variovorax paradoxus*]	95%	5.00E-51	60%	WP_057592616.1
rifampin ADP-ribosyl transferase [*Caulobacter vibrioides*]	89%	7.00E-51	62%	WP_058349090.1
rifampin ADP-ribosylating transferase [*Cytophaga hutchinsonii*]	98%	9.00E-51	56%	WP_011585690.1
rifampin ADP-ribosyl transferase [*Streptomyces purpeofuscus*]	100%	3.00E-50	57%	WP_063764233.1
rifampin ADP-ribosyl transferase [*Knoellia aerolata*]	93%	3.00E-50	61%	WP_052112740.1
rifampin ADP-ribosyl transferase [*Pontibacter roseus*]	91%	3.00E-50	59%	WP_018478319.1
rifampin ADP-ribosyl transferase [*Streptomyces kanamyceticus*]	90%	4.00E-50	62%	WP_055545547.1
rifampin ADP-ribosylating transferase [*Oscillatoria nigro-viridis*]	91%	4.00E-50	60%	WP_015175623.1
rifampin ADP-ribosylating transferase	92%	5.00E-50	59%	WP_009965045.1

[*Verrucomicrobium spinosum*]				
rifampin ADP-ribosylating transferase [*Clostridium bolteae*]	92%	5.00E-50	56%	WP_002571935.1
rifampin ADP-ribosyl transferase [*Clostridium neopropionicum*]	92%	5.00E-50	60%	WP_066091112.1
rifampin ADP-ribosyl transferase [*Pseudarthrobacter phenanthrenivorans*]	91%	6.00E-50	60%	WP_041652569.1
rifampin ADP-ribosyl transferase [*Myroides injenensis*]	97%	6.00E-50	56%	WP_010257556.1
rifampin ADP-ribosylating transferase [*Clostridium clostridioforme*]	92%	6.00E-50	56%	WP_002589901.1
rifampin ADP-ribosyl transferase [*Flavihumibacter solisilvae*]	89%	7.00E-50	59%	WP_039140159.1
ribosomal subunit interface protein [*Riemerella anatipestifer*]	97%	8.00E-50	56%	WP_064969801.1
rifampin ADP-ribosylating transferase ARR-2 [*Collimonas fungivorans*]	93%	8.00E-50	59%	WP_061540182.1
rifampin ADP-ribosyl transferase [*Spirosoma spitsbergense*]	91%	2.00E-49	60%	WP_063881410.1
rifampin ADP-ribosyl transferase [*Chryseobacterium angstadtii*]	92%	2.00E-49	57%	WP_048508524.1
rifampin ADP-ribosylating transferase [*Fluviicola taffensis*]	91%	4.00E-49	57%	WP_013687973.1
ADP-ribosyltransferase [*Nitratireductor pacificus*]	90%	6.00E-49	58%	WP_008599148.1
rifampin ADP-ribosyl transferase [*Spirosoma luteum*]	91%	1.00E-48	58%	WP_018621553.1
rifampin ADP-ribosyl transferase [*Chryseobacterium indologenes*]	89%	1.00E-48	58%	WP_062697472.1
rifampin ADP-ribosyl transferase [*Pseudarthrobacter sulfonivorans*]	92%	2.00E-48	58%	WP_058932937.1
rifampin ADP-ribosyl transferase [*Cytophaga aurantiaca*]	90%	2.00E-48	57%	WP_018342679.1
rifampin ADP-ribosyl transferase [*Phenylobacterium zucineum*]	90%	4.00E-48	60%	WP_041373627.1
rifampin ADP-ribosylating transferase [*Desulfotomaculum hydrothermale*]	100%	4.00E-48	54%	WP_008413283.1
rifampin ADP-ribosyl transferase [*Luteibacter rhizovicinus*]	92%	4.00E-48	56%	WP_063572277.1
hypothetical protein [*Chryseobacterium soli*]	92%	4.00E-48	54%	WP_034710413.1
hydrolase or acyltransferase of alpha/beta superfamily protein [*Arthrobacter nitrophenolicus*]	94%	7.00E-48	56%	WP_009358747.1
rifampin ADP-ribosyl transferase [*Devosia riboflavina*]	88%	7.00E-48	58%	WP_035078136.1
rifampin ADP-ribosyl transferase [*Leptolyngbya boryana*]	89%	7.00E-48	58%	WP_026148669.1
rifampin ADP-ribosyl transferase [*Chryseobacterium vrystaatense*]	93%	7.00E-48	55%	WP_034749533.1
hypothetical protein [*Runella zeae*]	90%	8.00E-48	57%	WP_028668032.1
ribosomal subunit interface protein [*Elizabethkingia anophelis*]	100%	9.00E-48	51%	WP_035590937.1

hypothetical protein [*Chryseobacterium luteum*]	93%	9.00E-48	55%	WP_034702130.1
rifampin ADP-ribosyl transferase [*Sunxiuqinia dokdonensis*]	90%	1.00E-47	58%	WP_053187313.1
NAD(+)-rifampin ADP-ribosyltransferase Arr-4 [*Pseudomonas aeruginosa*]	91%	2.00E-47	57%	WP_033959319.1
rifampin ADP-ribosyl transferase [*Rudanella lutea*]	99%	2.00E-47	52%	WP_019990724.1
rifampin ADP-ribosylating transferase ARR-2 [*Collimonas pratensis*]	93%	2.00E-47	58%	WP_061939301.1
rifampin ADP-ribosyl transferase [*Nocardia inohanensis*]	92%	3.00E-47	58%	WP_067813926.1
rifampin ADP-ribosyl transferase [*Caulobacter henricii*]	89%	4.00E-47	60%	WP_035041762.1
rifampin ADP-ribosyl transferase [*Pseudoxanthomonas suwonensis*]	86%	5.00E-47	60%	WP_037061093.1
ribosomal subunit interface protein [*Staphylococcus xylosus*]	94%	1.00E-46	56%	WP_039067545.1
rifampin ADP-ribosyl transferase [*Runella slithyformis*]	89%	1.00E-46	55%	WP_013930076.1
hypothetical protein [*Anaeromyxobacter dehalogenans*]	88%	3.00E-46	60%	WP_049760209.1
ribosomal subunit interface protein [*Herbaspirillum hiltneri*]	90%	3.00E-46	55%	WP_053195305.1
ribosomal subunit interface protein [*Paucisalibacillus globulus*]	93%	3.00E-46	56%	WP_026909123.1
ribosomal subunit interface protein [*Rufibacter roseus*]	88%	4.00E-46	53%	WP_066621345.1
hypothetical protein [*Enterococcus raffinosus*]	90%	5.00E-46	54%	WP_010747320.1
ribosomal subunit interface protein [*Flexithrix dorotheae*]	89%	6.00E-46	57%	WP_063745009.1
rifampin ADP-ribosyl transferase [*Dokdonella koreensis*]	88%	6.00E-46	56%	WP_067643213.1
rifampin ADP-ribosyl transferase [*Sphingomonas sanxanigenens*]	88%	6.00E-46	59%	WP_039997119.1
rifampin ADP-ribosyl transferase [*Mucilaginibacter gotjawali*]	90%	1.00E-45	56%	BAU55424.1
rifampin ADP-ribosyl transferase [*Clostridium saccharoperbutylacetonicum*]	90%	2.00E-45	55%	WP_015392137.1
rifampin ADP-ribosylating transferase ARR-2 [*Klebsiella pneumoniae*]	90%	2.00E-45	57%	KYL72167.1
ADP-ribosylating transferase [*Vibrio fluvialis*]	91%	2.00E-45	57%	ADK38648.1

By using the amino acid sequence of the rifampicin ADP-ribosyltransferase from *Mycobacterium smegmatis* mc^2155 (Accession: ABK71231) as a query, the ADP-ribosyltransferase-like proteins of non-mycobacterial species were identified by systematic BLASTP (non-redundant protein sequences, E-value <0.001). The first 100 non-mycobacterial species with the best hits predicted to possess ADP-ribosyltransferase-like proteins are listed.

ADDENDUM

Personal contribution to chapter 1

My contribution as first author to this manuscript was as follows:

- Design of the study.
- Analysis of the RpoB rifampicin resistance sequence clusters from *M. abscessus.*
- Distribution analysis of ADP-ribosyltransferase-like proteins in selected mycobacterial species and in non-mycobacterial species.
- Heterologous expression of *MAB_0591* in *M. tuberculosis.*
- Generation of *M. abscessus MAB_0591* deletion mutant.
- DST of *M. abscessus* Δ*MAB_0591.*
- DST of C25 modified rifamycin derivatives.
- Writing of the manuscript.
- Revision of the manuscript.

CHAPTER 2

Elucidation of *Mycobacterium abscessus* aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes

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ABSTRACT

Objectives: *Mycobacterium abscessus* is innately resistant towards a variety of drugs thereby limiting therapeutic options. Bacterial resistance towards aminoglycosides (AGs) mainly is conferred by AG-modifying enzymes which often have overlapping activities. Several putative AG-modifying enzymes are encoded in the genome of *M. abscessus*. The aim of this study is to investigate the molecular basis underlying AG resistance in *M. abscessus*.

Methods: *M. abscessus* deletion mutants deficient in either of three genes potentially involved in AG resistance, *aac(2')*, *eis1* and *eis2*, were generated by targeted gene inactivation, as were combinatorial double and triple deletion mutants. Minimal inhibitory concentration (MIC) assays were used to study susceptibility towards a variety of AG drugs and towards capreomycin.

Results: Deletion of *aac(2')* increased susceptibility of *M. abscessus* towards kanamycin B, tobramycin, dibekacin and gentamicin C. Deletion of *eis2* increased susceptibility towards capreomycin, hygromycin B, amikacin and kanamycin B. Deletion of *eis1* did not affect drug susceptibility. Equally low MICs in wildtype and mutant strains for apramycin, arbekacin, isepamicin and kanamycin A indicate that these drugs are not inactivated by either AAC(2') or Eis enzymes.

Conclusions: *M. abscessus* expresses two distinct AG resistance determinants AAC(2') and Eis2 which confer clinically relevant drug resistance.

INTRODUCTION

Mycobacterium abscessus is a rapid growing mycobacterium (RGM) of increasing medical importance. This emerging pathogen causes bronchopulmonary infections in individuals with cystic fibrosis (CF)[1] and chronic pulmonary disease, like pneumoconiosis and bronchiectasis.[2] It also causes severe infections following surgery, transplantation, tattooing and mesotherapy.[3-8] Treatment of an *M. abscessus* infection is difficult due to the bacteria's high degree of intrinsic resistance to chemotherapeutic agents.[9] The pathogen is naturally resistant towards many major classes of antibiotics used for treatment of Gram-positive and Gram-negative bacterial infections, like β-lactams, aminoglycosides (AGs) [kanamycin (B), gentamicin C] and macrolides (erythromycin). In addition, it is also resistant towards first-line tuberculosis drugs, e.g. isoniazid and rifampicin. *M. abscessus* has been called an antibiotic nightmare since treatment options against *M. abscessus* infection are more limited than for *Mycobacterium tuberculosis* infection.[10] While no standard treatment recommendations for pulmonary *M. abscessus* infections have yet been established, current guidelines propose administration of an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to macrolides and the intravenous AG amikacin in combination with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[11] AGs and macrolides inhibit protein biosynthesis by binding to the small and large ribosomal subunit, respectively. Clinically acquired pan-AG and pan-macrolide resistance has been attributed to mutations in ribosomal RNA genes *rrs* and *rrl*, coding for 16S and 23S rRNA, respectively. Due to the presence of a single ribosomal RNA (*rrn*) operon, corresponding resistance mutations have readily been observed in *M. abscessus*.[12-14] However, distinct mechanisms have been proposed to be responsible for innate AG resistance in *M. abscessus*.[9,15]

AG antibiotics form a group of hydrophilic molecules, consisting of a characteristic, central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bond(s). They inhibit prokaryotic protein biosynthesis by binding to the A-site of the 16S rRNA. AGs are active against a wide range of aerobic Gram-negative bacilli, staphylococci and mycobacteria. Resistance towards AGs is due to low uptake, increased efflux, target modification or enzyme mediated drug modification. Originally isolated from microorganisms (mainly *Streptomyces spp*.), semisynthetic derivatives of AGs have been generated to improve pharmacological properties of the drugs and to overcome bacterial resistance mechanisms.[16] The semisynthetic amikacin is a cornerstone of *M. abscessus* infection therapy, while other AGs are not frequently used. *M. abscessus* genome annotation suggests the presence of various AG-

modifying enzymes such as AG phosphotransferases (APH), AG nucleotidyltransferases (ANT) and AG acetyltransferases (AAC).[17] The individual contribution of these genes to *M. abscessus* AG susceptibility can hardly be predicted due to overlapping specificities.

Elucidation of gene functions, drug target and host-pathogen interaction heavily relies on generation of isogenic mutants. Usually, antibiotic resistance markers are used for primary selection of transformants. Tools for genetic manipulation of mycobacteria in general and *M. tuberculosis* in particular, have been developed during the past 2-3 decades.[18] In contrast, attempts for genetic manipulation of *M. abscessus* have been reported to be very difficult and often were unsuccessful[19-21], although some progress has been made.[22-25] We recently developed genetic tools for *M. abscessus*[26] and now exploited them for elucidating intrinsic AG resistance mechanisms in the *M. abscessus* ATCC 19977 type strain. We particularly addressed the role of putative AG acetyltransferases on *M. abscessus* AG and capreomycin resistance. We generated single, double and triple mutants of *M. abscessus* by targeted deletion of three genes potentially involved in AG resistance and characterized those mutants by phenotypic drug susceptibility testing. Interestingly, a double mutant proved to be susceptible towards a wide variety of AGs and towards the peptide antibiotic capreomycin.

MATERIALS AND METHODS

Bacterial strains and media

The *Escherichia coli* laboratory strain XL1-Blue MRF' (Stratagene, Switzerland) and ER2925 *dam*$^-$, *dcm*$^-$ were used for cloning and propagation of plasmids. The strains were grown in Luria-Bertani (LB) medium containing either of the antibiotics: Ampicillin (120 mg/L), kanamycin A (50 mg/L), apramycin (50 mg/L), and hygromycin B (100 mg/L). Antibiotics (Sigma-Aldrich, Switzerland) were dissolved in H_2O according to the manufacturer's recommendations and stored as a stock solution until usage.

M. abscessus ATCC 19977 were grown in Middlebrook 7H9-OADC-Tween 80 or LB containing either of the antibiotics, if required: kanamycin A (50 mg/L), apramycin (50 mg/L), isoniazid (32 mg/L). For preparation of electrocompetent cells two cell culture flasks each containing 200 mL 7H9-OADC-Tween 80 were inoculated 1:100, incubated at 37 °C and gently shaken daily. When OD_{600} reached 0.4-0.8, cultures were set on ice for 90 minutes. Bacteria were harvested by centrifugation, and repeatedly resuspended in ice-cold glycerol

(10% v/v) while gradually reducing the volume.[27] Finally, bacteria were resuspended in 2 mL glycerol (10% v/v) and either frozen in liquid nitrogen or directly used for electroporation, as we recently described.[26] Genomic DNA was isolated by phenol-chloroform-isoamyl alcohol extraction, as described previously.[28]

Generation of vectors

The apramycin resistance cassette from plasmid pSET152[29] was PCR amplified (primers Apr_F and Apr_R) (see Table S1 for list of primers) and cloned as 1-kbp *Spe*I fragment into *Spe*I-digested plasmid pMV361[30] to result in plasmid pMV361-*apr*. An *M. tuberculosis katG* fragment (2.8-kbp) was PCR amplified (primers KatG_F and KatG_R), saved into pGEM-T-easy to result in pGEM-T-*katG*. Subsequently, the 2.8-kbp *Swa*I-*katG*-*Hpa*I fragment was cloned into *Hpa*I-linearized pMV361-*apr* to result in pMV361-*apr-katG*. Removal of mycobacteriophage *attP*/*int* from plasmid pMV361-*apr-katG* by *Xba*I excision and self-ligation of the vector backbone resulted in the prototype suicide vector pSE-*apr-katG* into which flanking regions of the target genes were cloned.

Disruption of *MAB_4395* [*aac(2')*] in *M. abscessus*

A 1.6-kbp *Nde*I/*Nhe*I fragment of *M. abscessus* ATCC 19977 from position 4477184 to 4478794 comprising the 5′ flanking sequence and a 1.5-kbp *Nhe*I/*Mlu*I fragment from position 4479125 to 4480674 comprising the 3′ flanking sequence of *MAB_4395* [*aac(2')*] were PCR amplified (primers 4395_UP_F, 4395_UP_R and 4395_DW_F, 4395_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Subsequently, fragments were ligated stepwise into saving vector pMCS5 with respective enzymes resulting in sub-cloning vector pMCS5-*MAB_4395*. The region comprising 5′ and 3′ flanking sequences of *aac(2')* was cut out with *Stu*I and cloned into *Hpa*I linearized pSE-*apr-katG* vector to result in knockout vector pSE-Δ*aac(2')*. The *aac(2')* allele was deleted from *M. abscessus* ATCC 19977 chromosome using apramycin for positive and isoniazid for negative selection. Deletion of *aac(2')* was confirmed by Southern blot analysis of *Van91*I digested genomic DNA with a 0.3-kbp 5′ *aac(2')* probe amplified with primers P_4395_F and P_4395_R. A 2.2-kbp fragment from *M. abscessus* ATCC 19977 from position 4477131 to 4479338 spanning the entire *aac(2')* gene was amplified with primers C_4395_F and C_4395_R and cloned into

*Hind*III digested pMV361 vector to result in pMV361-*aac(2')* complementation vector. *M. abscessus* Δ*aac(2')* was transformed with pMV361-*aac(2')* and control vector pMV361, respectively. Genetic complementation was confirmed by Southern blot analysis with the same 5′ *aac(2')* probe.

Disruption of *MAB_4124* (*eis1*) in *M. abscessus*

A 1.6-kbp *Apa*I/*Nde*I fragment of *M. abscessus* ATCC 19977 from position 4187416 to 4189052 comprising the 5′ flanking sequence and a 1.7-kbp *Nde*I/*Nhe*I fragment from position 4189857 to 4191580 comprising the 3′ flanking sequence of *MAB_4124* (*eis1*) were PCR amplified (primers 4124_UP_F, 4124_UP_R and 4124_DW_F, 4124_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Fragments were stepwise cloned with corresponding enzymes into saving vectors and subsequently into *Not*I/*Xba*I digested vector pSE-*apr-katG* to result in knockout vector pSE-Δ*eis1*. The *eis1* allele was deleted from the *M. abscessus* ATCC 19977 chromosome using apramycin positive and isoniazid negative selection. Deletion was confirmed by Southern blot analysis using *Van91*I digested genomic DNA and a 0.3-kbp 5′ *eis1* fragment amplified with primers P_4124_F and P_4124_R.

Disruption of *MAB_4532c* (*eis2*) in *M. abscessus*

A 1.8-kbp *Hpa*I/*Pfl23*II fragment of *M. abscessus* ATCC 19977 from position 4612843 to 4614693 comprising the 5′ flanking sequence and a 1.5-kbp *Pac*I/*Pfl23*II fragment from position 4615391 to 4616869 comprising the 3′ flanking sequence of *MAB_4532c* (*eis2*) were PCR amplified (primers 4532c_UP_F, 4532c_UP_R and 4532c_DW_F, 4532c_DW_R) using genomic DNA from *M. abscessus* ATCC 19977. Fragments were stepwise cloned into vector pSE-*apr-katG* resulting in knockout vector pSE-Δ*eis2*. The *eis2* allele was deleted from *M. abscessus* ATCC 19977 chromosome using apramycin positive and isoniazid negative selection. Deletion was confirmed by Southern blot analysis with *Van91*I digested genomic DNA and 0.3-kbp 5′ *eis2* probe amplified with primers P_4532c_F and P_4532c_R. For complementation a 1.3 kbp fragment from *M. abscessus* ATCC 19977 (position 4614320 to 4615651) spanning the entire *eis2* gene was PCR amplified (primers C_4532c_F, C_4532c_R) and cloned via *Hind*III into pJB-*apr* vector to result in pJB-*apr-eis2* complementation vector. *M. abscessus* Δ*eis2* was transformed with pJB-*apr-eis2* and control

vector pJB-*apr*, respectively. Genetic complementation was confirmed by Southern blot analysis.

MIC assays

Kanamycin B, tobramycin, dibekacin, arbekacin, gentamicin C [C1 (<45%), C2 (<35%) and C1a (<30%)], isepamicin, amikacin, kanamycin A, apramycin, streptomycin, hygromycin B and capreomycin were bought from Sigma-Aldrich, CH. Antibiotics were dissolved in H_2O according to the manufacturer's recommendations, were filter sterilized, aliquoted into stock solutions and stored at -20 °C. MIC determination for *M. abscessus* strains was done according to CLSI guidelines[31] and basically as we previously described.[26]

RESULTS

Targeted inactivation of putative AG resistance genes of *M. abscessus*

Genome annotation[17], genome analysis and AG drug susceptibility testing[15] suggest the presence of several putative AG acetyltransferases in *M. abscessus*. Open reading frame *MAB_4395* is annotated as a putative AG 2′-*N*-acetyltransferase [*aac(2′)*][17], while *MAB_4124* and *MAB_4532c* show homology to *eis*. Eis (Enhanced intracellular survival) proteins are found in a variety of mycobacterial and non-mycobacterial species.[32-33] Overexpression of Eis (Rv2416c) confers increased kanamycin resistance to *M. tuberculosis*.[34] *M. abscessus MAB_4124* is the closest homologue of *M. tuberculosis Rv2416c* (33% identity), which we therefore name *eis1*. Interestingly, a second Eis homologue encoded by *MAB_4532c* is present in the *M. abscessus* genome. In a phylogenetic tree constructed from 29 Eis homologues[33], MAB_4532c (which we name Eis2) is clustered with Eis homologues from the non-mycobacterial clade instead of the mycobacterial group, for instance *M. abscessus* Eis2 shows 23% identity to *Anabaena variabilis* Eis. We addressed the role of the three acetyltransferases in *M. abscessus* AG resistance by engineering and characterizing corresponding unmarked deletion mutants in *M. abscessus* type strain ATCC 19977.

Table 1. Strains used in this study

Strain	Description	Source
E. coli XL1-Blue MRF'	Cloning and propagation of plasmids	Stratagene
E. coli ER2925 *dam*$^-$, *dcm*$^-$	Cloning and propagation of plasmids with methylase susceptible restriction enzymes	New England BioLabs
M. abscessus ATCC 19977	*M. abscessus* type strain	(Ripoll *et al*., 2009)[17]
M. abscessus Δ*aac(2')*	*M. abscessus aac(2')* deletion mutant; derivative of *M. abscessus* ATCC 19977	This study
M. abscessus Δ*eis1*	*M. abscessus eis1* deletion mutant; derivative of *M. abscessus* ATCC 19977	This study
M. abscessus Δ*eis2*	M. *abscessus eis2* deletion mutant; derivative of *M. abscessus* ATCC 19977	This study
M. abscessus Δ*aac(2')* Δ*eis1*	*M. abscessus aac(2') eis1* double deletion mutant; derivative of *M. abscessus* Δ*eis1*	This study
M. abscessus Δ*aac(2')* Δ*eis2*	*M. abscessus aac(2') eis2* double deletion mutant; derivative of *M. abscessus* Δ*aac(2')*	This study
M. abscessus Δ*eis1* Δ*eis2*	*M. abscessus eis1 eis2* double deletion mutant; derivative of *M. abscessus* Δ*eis2*	This study
M. abscessus Δ*aac(2')* Δ*eis1* Δ*eis2*	*M. abscessus aac(2') eis1 eis2* triple deletion mutant; derivative of *M. abscessus* Δ*eis1* Δ*eis2*	This study
M. abscessus Δ*aac(2')* pMV361-*aac(2')*	*M. abscessus aac(2')* mutant transformed with complementation vector pMV361-*aac(2')*	This study
M. abscessus Δ*aac(2')* pMV361	*M. abscessus aac(2')* mutant transformed with pMV361 control vector	This study
M. abscessus Δ*eis2* pJB-*apr-eis2*	*M. abscessus* Δ*eis2* mutant transformed with complementation vector pJB-*apr-eis2*	This study
M. abscessus Δ*eis2* pJB-*apr*	*M. abscessus* Δ*eis2* mutant transformed with control vector pJB-*apr*	This study

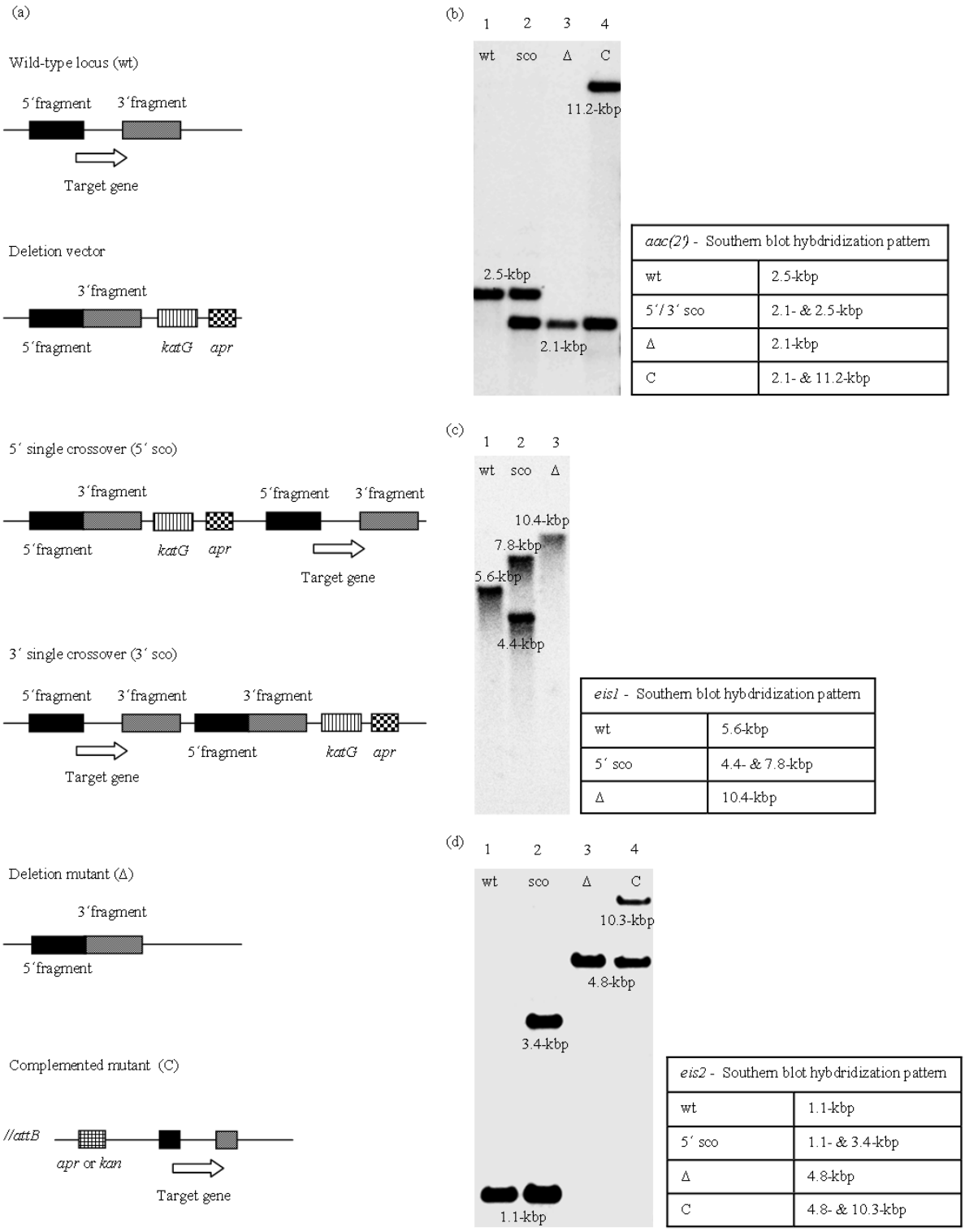
Flanking fragments of the target genes *aac(2')*, *eis1* and *eis2* were cloned into plasmid pSE-*apr-katG* to result in suicide vectors pSE-Δ*aac*, pSE-Δ*eis1* and pSE-Δ*eis2*, respectively. These flanking fragments enable homologous recombination of the suicide vector. The apramycin resistance cassette of the vector backbone facilitates selection of single crossover transformants resulting from vector integration by intermolecular homologous recombination at the target locus. Catalase-peroxidase KatG, the activator of the tuberculosis prodrug isoniazid[35], serves as a negative selectable marker since it sensitizes *M. abscessus* to isoniazid. Expression of KatG facilitates screening for deletion mutants resulting from resolving the integration of the suicide vector by a second intramolecular homologous recombination.[26] Electrocompetent *M. abscessus* were transformed and apramycin selection and Southern blot analysis were applied to identify single crossover transformants. Subsequently, isoniazid counterselection and Southern blot analysis were applied for the identification of *M. abscessus* Δ*aac(2')*, *M. abscessus* Δ*eis1* and *M. abscessus* Δ*eis2* mutants (Figure 1). Deletion mutants showing a phenotype [*M. abscessus* Δ*aac(2')* and *M. abscessus*

Δ*eis2*] were transformed with single-copy, i.e. pMV361-based complementation and control vectors, respectively (see Table 1 for list of strains and Table 2 for list of plasmids). Furthermore, single deletion mutants served as parental strains for construction of double deletion mutants by transformation with a second targeting vector. Double deletion mutants, *M. abscessus* Δ*aac(2')* Δ*eis1*, *M. abscessus* Δ*aac(2')* Δ*eis2* and *M. abscessus* Δ*eis1* Δ*eis2*, were confirmed by Southern blot analyses. Finally, by transformation of pSE-Δ*aac(2')* into *M. abscessus* Δ*eis1* Δ*eis2*, the triple mutant *M. abscessus* Δ*aac(2')* Δ*eis1* Δ*eis2* was constructed (Figure 2).

Table 2. Plasmids used in this study

Plasmid	**Description; selectable marker**	**Source**
pMV361	Integrative *E. coli*/mycobacterial shuttle vector; Kan^R	(Stover *et al*., 1991)[30]
pSET152	*Streptomyces* vector with apramycin resistance gene [*aac(3)IV*], template for *apr* amplification; Apr^R	(Wilkinson *et al*., 2002)[29]
pGEM-T-easy	PCR saving vector; Amp^R	Promega
pMCS5	General cloning vector; Amp^R	MBio
pGEM-T-katG	Intermediate vector for *katG* subcloning; Amp^R	This study
pMV361-*apr*	Integrative *E. coli*/mycobacterial shuttle vector; Kan^R, Apr^R	This study
pMV361-*apr-katG*	Derivative of pMV361-*apr* containing *M. tuberculosis katG*; Kan^R, Apr^R, INH^S	This study
pSE-*apr-katG*	Derivative of pMV361-*apr-katG* deleted for mycobacteriophage integrase *int* and *attP*; intermediate vector used for cloning of Δ*aac(2')*, Δ*eis1* and Δ*eis2* alleles; Kan^R, Apr^R, INH^S	This study
pMCS5-*MAB_4395*	Intermediate vector containing *aac(2')* flanking regions; Amp^R	
pSE-Δ*aac(2')*	Suicide vector; derivative of pSE-*apr-katG* carrying Δ*aac(2')* allele; Apr^R, Kan^R, INH^S	This study
pSE-Δ*eis1*	Suicide vector; derivative of pSE-*apr-katG* carrying Δ*eis1* allele; Apr^R, Kan^R, INH^S	This study
pSE-Δ*eis2*	Suicide vector; derivative of pSE-*apr-katG* carrying Δ*eis2* allele; Apr^R, Kan^R, INH^S	This study
pMV361-*aac(2')*	Integrative complementation vector for *aac(2')*, Kan^R	This study
pJB-*apr*	Derivative of pMV361 in which the kanamycin resistance cassette was substituted by the apramycin resistance cassette; Apr^R	This study
pJB-*apr-eis2*	Integrative complementation vector for *eis2*; derivative of pJB-*apr*; Apr^R	This study

Kan^R = kanamycin resistance cassette; Apr^R = apramycin resistance cassette; Amp^R = ampicillin resistance cassette; INH^S = isoniazid susceptibility cassette



aac(2') - Southern blot hybdridization pattern

wt	2.5-kbp
5´/3´ sco	2.1- & 2.5-kbp
Δ	2.1-kbp
C	2.1- & 11.2-kbp

eis1 - Southern blot hybdridization pattern

wt	5.6-kbp
5´ sco	4.4- & 7.8-kbp
Δ	10.4-kbp

eis2 - Southern blot hybdridization pattern

wt	1.1-kbp
5´ sco	1.1- & 3.4-kbp
Δ	4.8-kbp
C	4.8- & 10.3-kbp

Figure 1. Genotypic analyses of *M. abscessus* deletion mutants. (a) General schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirms the deletion of *aac(2')* (*MAB_4395*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1), *M. abscessus* transformant with pSE-Δ*aac(2')* targeting vector prior to (2) and after KatG-dependent isoniazid-counterselection (3) and after transformation of counterselected mutant with pMV361-*aac(2')* complementation vector (4) was digested with *Van91*I and probed with a fragment from the *5′ aac(2')* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 2.5-kbp fragment of the wild-

type (wt) parental strain, to the 2.1-kbp and 2.5-kbp fragments after site-specific homologous recombination (single crossover; sco), to a 2.1-kbp fragment of the *M. abscessus* Δ*aac(2')* mutant (Δ) and to the 2.1-kbp and 11.2-kbp fragments of the *M. abscessus* Δ*aac(2')* pMV361-*aac(2')* complemented mutant strain (C). (c) Southern blot analysis confirms the deletion of *eis1* (*MAB_4124*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1), *M. abscessus* transformant with pSE-Δ*eis1* targeting vector prior to (2) and after KatG-dependent isoniazid-counterselection (3) was digested with *Van91*I and probed with a fragment from the 5′ *eis1* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 5.6-kbp fragment of the wild-type (wt) parental strain, to the 4.4-kbp and 7.8-kbp fragments after site-specific homologous recombination (sco) and to a 10.4-kbp fragment of the *M. abscessus* Δ*eis1* mutant (Δ). (d) Southern blot analysis confirms the deletion of *eis2* (*MAB_4532c*) from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1), *M. abscessus* transformant with pSE-Δ*eis2* targeting vector prior to (2) and after KatG-dependent isoniazid-counterselection (3) and after transformation of counterselected mutant with pJB-*apr-eis2* complementation vector (4) was digested with *Van91*I and probed with a fragment from the 5′ *eis2* flanking region. Based on *M. abscessus* genome annotation and vector sequence, the pattern is consistent with hybridization to a 1.1-kbp fragment of the wild-type (wt) parental strain, to the 1.1-kbp and 3.4-kbp fragments after site-specific homologous recombination (sco), to a 4.8-kbp fragment of the *M. abscessus* Δ*eis2* mutant (Δ) and to the 4.8-kbp and 10.3-kbp fragments of the *M. abscessus* Δ*eis2* pJB-*apr-eis2* complemented mutant strain (C).

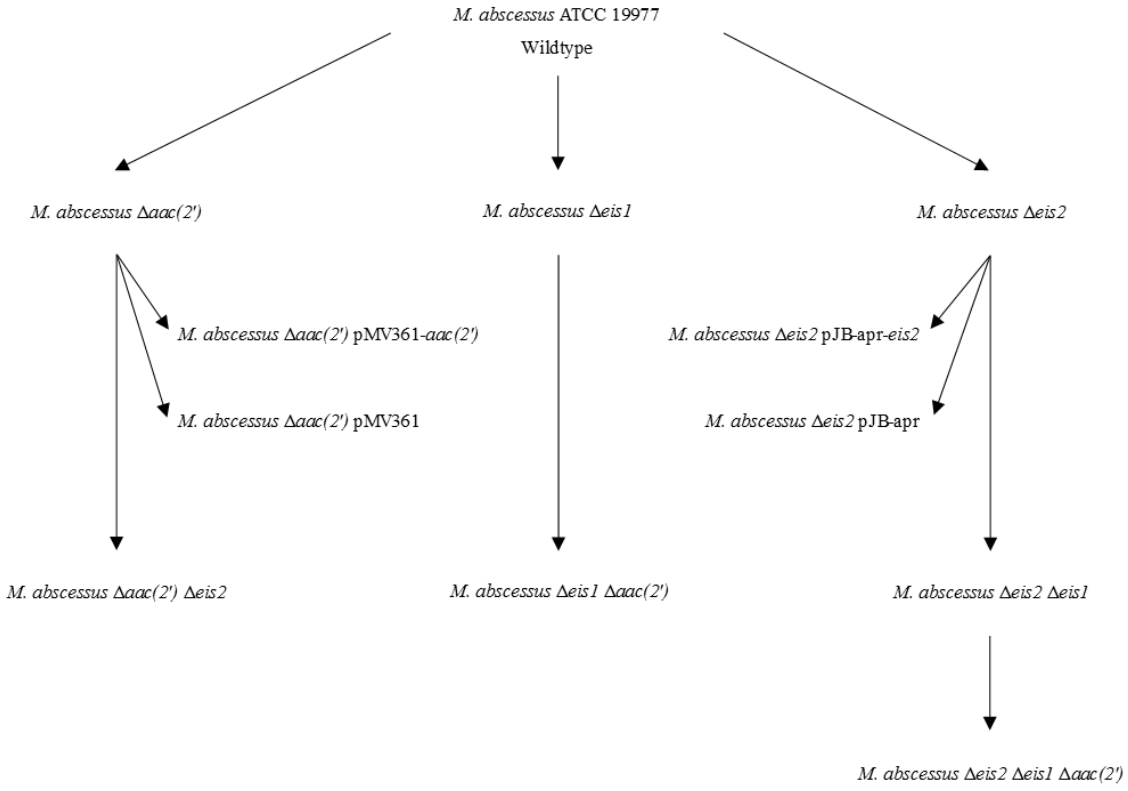


Figure 2. Genealogy of *M. abscessus* strains used in this study for the construction of single, double and triple deletion mutants.

Drug susceptibility testing of *M. abscessus* mutant strains

Drug susceptibility of *M. abscessus* strains towards a variety of structurally similar 4,6-distubstituted AGs (Figure 3) was tested in Cation Adjusted Müller-Hinton Broth. In addition, drug susceptibility towards structurally atypical AGs apramycin, hygromycin B and streptomycin and towards the peptide antibiotic capreomycin (also inhibiting protein biosynthesis by interaction with 16S rRNA A-site) was determined. Growth was judged after 3, 5, 7 and 12 days of incubation and the MICs were determined. The median MIC values on day 5 are listed in Table 3. The median MIC values read at all time points are shown in Table S2. A variety of mutant phenotypes was observed. Deletion of *aac(2')* increased susceptibility of *M. abscessus* towards kanamycin B (64-fold), tobramycin (32-fold), dibekacin (16-fold), gentamicin C (4-fold). Deletion of *aac(2')* did not affect MIC towards arbekacin, isepamicin, amikacin, kanamycin A, apramycin, streptomycin, hygromycin B and capreomycin (Table 3). The tobramycin, dibekacin and gentamicin C wild-type phenotype was restored upon transformation with the complementation vector pMV361-*aac(2')*, but not by transformation with the backbone control vector pMV361 (Table S2). The presence of a kanamycin resistance cassette [*aph(3')*] in the backbone of the complementation vector interfered with DST for kanamycin A and B and MICs for these compounds are therefore not reported. Deletion of *eis1* did not result in a detectable phenotype; MIC towards none of the tested antibiotics was altered. Due to the absence of a phenotype, no complementation vector was constructed. Deletion of *eis2* increased susceptibility of *M. abscessus* towards several AGs. However, the susceptibility pattern of *M. abscessus* Δ*eis2* clearly differed from that of *M. abscessus* Δ*aac(2')*. Deletion of *eis2* increased susceptibility towards kanamycin B (4-fold), amikacin (8-fold), hygromycin B (16-fold) and capreomycin (32-fold). Deletion of *eis2* did not affect susceptibility towards tobramycin, dibekacin, arbekacin, gentamicin C, isepamicin, kanamycin A, apramycin, and streptomycin. Transformation of the *Δeis2* mutant with the complementation vector pJB-*apr-eis2*, but not with the vector backbone pJB-*apr*, restored wild-type MIC levels towards amikacin, hygromycin B and capreomycin (Table S2). The presence of the apramycin resistance cassette in the backbone of the complementation vector interfered with DST for kanamycin (A and B), tobramycin, dibekacin, gentamicin C and apramycin, and therefore MIC values for these antibiotics are not reported. Double mutant *M. abscessus* Δ*aac(2')* Δ*eis1* had the same phenotype as the Δ*aac(2')* single mutant. Double mutant *M. abscessus* Δ*eis1* Δ*eis2* had the same phenotype as the Δ*eis2* single mutant. The *M. abscessus* Δ*aac(2')* Δ*eis2* double mutant showed increased susceptibility towards kanamycin

B, tobramycin, dibekacin, gentamicin C, amikacin, hygromycin B and capreomycin, while MICs towards arbekacin, isepamicin, kanamycin A, apramycin and streptomycin were not altered. Thus, the phenotype of the Δ*aac(2')* Δ*eis2* double mutant is a combination of the phenotype of the single mutants. The phenotype of the triple Δ*aac(2')* Δ*eis1* Δ*eis2* mutant was similar to the phenotype of the Δ*aac(2')* Δ*eis2* double mutant. Together, these data indicate a distinct role of *aac(2')* and of *eis2* in intrinsic AG and capreomycin resistance in *M. abscessus*. Notably, AAC(2') and Eis2 prevent antibacterial effects of different subset of AGs. The only exception is kanamycin B that is modified by both AAC(2') and Eis2. However, the deletion of *aac(2')* had a stronger effect on kanamycin B susceptibility than deletion of *eis2* (64-fold vs. 4-fold). AAC(2') is more influential than Eis2, since the MIC of the double deletion mutant is not further decreased as compared to the *aac(2')* deletion mutant. Deletion of *eis1* from the genome of the Δ*aac(2')* Δ*eis2* mutant did not further increase AG susceptibility, indicating a negligible role of Eis1 in *M. abscessus* intrinsic AG resistance even in an *aac(2') eis2* double deletion mutant.

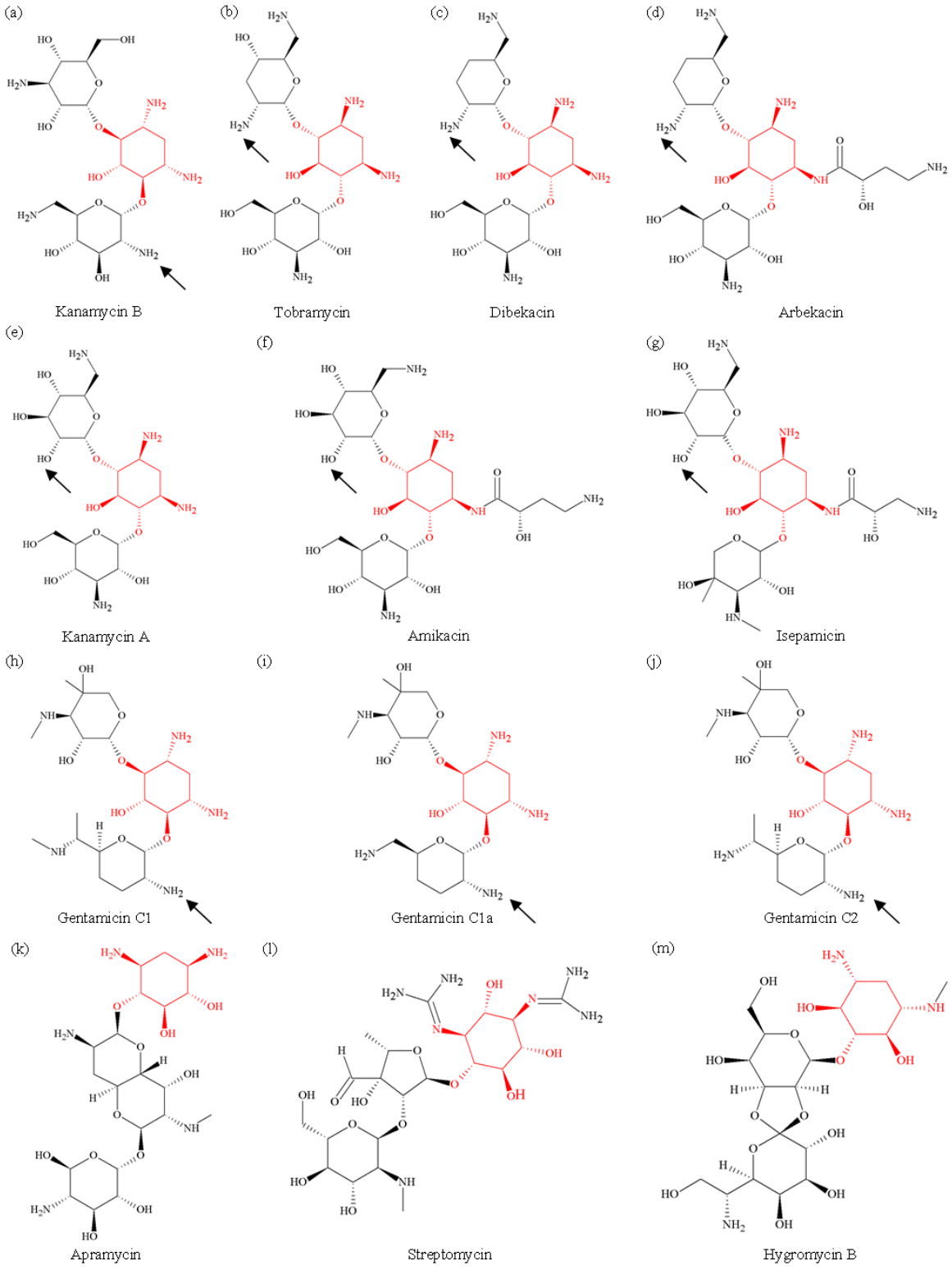


Figure 3: Structure of 4,6-disubstituted (a-j) and atypical (k-m) AGs used in this study. The aminocyclitol ring is shown in red. Black arrows show the 2′-groups of the 4,6-distubstituted AGs (a-j).

Table 3. MIC determination of *M. abscessus* deletion mutants (median, day 5) [a,b]

Compound	Strain							
	Wildtype	*Δaac(2')*	*Δeis1*	*Δeis2*	*Δaac(2') Δeis1*	*Δaac(2') Δeis2*	*Δeis1 Δeis2*	*Δaac(2') Δeis1 Δeis2*
	Day 5 MIC (mg/L)							
Kanamycin B	8	0.125	8	2	0.125	0.125	1	0.125
Tobramycin	8	0.25	8	4	0.25	0.125	2	0.125
Dibekacin	16	1	16	16	2	1	16	1
Arbekacin	1	0.5	1	1	0.5	0.5	0.5	0.5
Gentamicin C	4	1	4	4	1	1	4	1
Isepamicin	1	1	1	0.5	1	0.5	0.5	0.5
Amikacin	2	2	2	0.25	2	0.25	0.25	0.25
Kanamycin A	1	1	1	0.5	1	0.5	0.5	0.25
Apramycin	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5
Streptomycin	32	32	32	32	32	32	32	32
Hygromycin B	256	256	256	16	256	16	16	16
Capreomycin	128	128	256	4	128	4	4	4

[a]For a complete overview of MIC results judged at days 3, 5, 7, 12 see Table S2
[b]For phenotype of single deletion mutants transformed with complementation vectors and control vectors also see Table S2

DISCUSSION

M. abscessus is a pathogen of increasing medical importance particularly in individuals with chronic pulmonary disease. The high level of intrinsic resistance towards many classes of antibiotics restricts antibiotic therapy.[9] Genome annotation[17], DST and biochemical assays predicted a role for a 2'-*N*-acetyltransferase in *M. abscessus* AG resistance.[15] A candidate gene eventually responsible for the increased MIC levels towards 2'-amino-AGs, *MAB_4395* [*aac(2')*] was inactivated by targeted gene deletion. Comparison of wildtype and mutant strain by phenotypic DST demonstrated a decreased MIC of the mutant towards a variety of drugs characterized by a 2'-amino-group (kanamycin B, tobramycin, dibekacin, gentamicin C). Kanamycin A which differs from kanamycin B by a single structural feature (2'-OH-group instead of a 2'-amino-group) has a lower MIC against the wildtype than kanamycin B and its MIC is not further decreased by *aac(2')* deletion. These data corroborate the hypothesis that *MAB_4395* encodes a functional AG 2'-*N*-acetyltransferase which renders *M. abscessus* relatively resistant towards several AGs with a 2'-amino-group. Amikacin is protected from AAC(2') modification due to the presence of a OH-group at 2'-position. Actually, amikacin is a derivative of kanamycin A, but with an L-hydroxyaminobutyryl amide (L-HABA) side chain at the position 1 of the 2-deoxystreptamine core. Tobramycin (3'-deoxy-kanamycin B) is inert to modification by 3'-phosphotransferases, but susceptible towards modification by AAC(2'). This is indicated by the observation that the kanamycin B and tobramycin MICs are similarly decreased in the Δ*aac(2')* mutant strain as compared to the parental strain. Since tobramycin is not a substrate for the 3'-AG-phosphotransferase [APH(3')] (which is encoded on the backbone of the complementation vector pMV361-*aac(2')*] restoration of the tobramycin phenotype could be addressed by transformation with the complementation vector. Phenotypic complementation was observed indicating that deletion of *aac(2')* is responsible for increased tobramycin susceptibility of the mutant. MIC towards arbekacin is similarly low in wildtype and Δ*aac(2')* mutant and also similar to the MIC of dibekacin of the Δ*aac(2')* mutant, while the MIC of the wildtype towards dibekacin is much higher. Dibekacin (3',4'-dideoxy-kanamycin B) differs from arbekacin at position 1 of the 2-deoxystreptamine core; there arbekacin carries an L-HABA chain, which obviously protects from AAC(2') activity.

Gentamicin C is not a pure component, but a mixture of different subclasses (C1, C2 and C1a). C-type gentamicin subgroups carry a 2'-NH_2-group. In contrast, B-type gentamicin carries a 2'-OH-group. Isepamicin is a derivative of gentamicin B with an L-HABA side chain

at position 1 of the 2-deoxystreptamine core. The MIC of the wildtype towards isepamicin is lower than towards gentamicin C, while both compounds have similarly (low) MICs against the Δ*aac(2')* mutant strain. These findings may be explained by the enzymatic modification of gentamicin C by the AAC(2') activity present in the wild-type strain. Taken together, comparison of the MICs of wildtype and Δ*aac(2')* mutant indicates that AAC(2') confers resistance to drugs carrying a 2'-NH_2-group, unless the position 1 of the 2-deoxystreptamine core is modified with a L-HABA chain (compare: dibekacin, arbekacin). Neither deoxygenation at 3' nor di-deoxygenation at positions 3' and 4' (compare kanamycin B with tobramycin and dibekacin, respectively) prevent *M. abscessus* AAC(2')-mediated drug resistance mechanisms. Approximately 10-fold decrease in MIC towards gentamicin C and tobramycin and even higher decrease in MIC towards dibekacin were also seen upon genetic inactivation of *Mycobacterium smegmatis aac(2')*.[36]

The *M. abscessus* Δ*eis1* mutant did not show any phenotype and therefore does not contribute to AG resistance, at least not during *in vitro* growth. Expression studies and biochemical assays would be required to address the physiological and enzymatic function of Eis1. It may be hypothesized that promoter up mutations might increase AG resistance. This hypothesis could also be addressed by transformation of wild-type strains with multi-copy vectors containing *eis1*. However besides AAC(2')-mediated AG resistance, *M. abscessus* possess at least a second mechanism of AG resistance which is mediated by Eis2. The Δ*eis2* mutant showed increased susceptibility towards the peptide antibiotic capreomycin and towards a distinct and more heterogeneous group of AGs: kanamycin B, amikacin, hygromycin B. Eis proteins of different origin have been shown to have multiple AG- and capreomycin-acetylating activities, for example *M. tuberculosis* and *M. smegmatis* Eis tri-acetylate neamine in a sequential manner, first at the 2'-, then at the 6'-, and finally at the 1-position. The number of acetylations depends on the AG itself, but also on the biological origin of the enzyme. Up to four acetyl residues may be transferred to tobramycin by *M. tuberculosis* Eis and *M. smegmatis* Eis, respectively.[33,37-38] Hygromycin is mono- and di-acetylated by *M. smegmatis* and *M. tuberculosis* Eis, respectively. Apramycin is di-acetylated by *M. smegmatis*, but is not a substrate for *M. tuberculosis* Eis. *M. abscessus* Eis2 does not inactivate apramycin (identical MIC of wildtype and Δ*eis2* mutant), although this does not exclude that apramycin is an *M. abscessus* Eis2 substrate. Corresponding acetylations might just not affect antibacterial activity. A combined application of an AAC(2') inhibitor together with specific NH_2-AGs (e.g. kanamycin B and gentamicin C) could enhance the activity of these AGs. Administration of an Eis2 inhibitor could enhance the activity of capreomycin and amikacin, the latter being a

cornerstone for treatment of *M. abscessus* infections.[39] As opposed to several 2-deoxystreptamine AGs, streptomycin MICs in the mutant strains remained at the high wild-type MIC levels, pointing to a distinct streptomycin resistance mechanism in *M. abscessus*. Our study demonstrates that apramycin, an AG with little ototoxicity[40], arbekacin, isepamicin and kanamycin A exhibit excellent *in vitro* activities against *M. abscessus* ATCC 19977 type strain and that the activity of these drugs is not affected by AAC(2') and Eis proteins, respectively. Our data further support MIC testing of these aminoglycosides against a broader set of *M. abscessus* clinical isolates.[15] The results from the suggested *in vitro* studies may provide a rational basis for designing clinical trials aiming at implementation of improved treatment regimens against one of the most drug resistant pathogens, *M. abscessus*.

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SUPPLEMENTARY DATA

Table S1. Primers used in this study

Name	Description	Sequence
Apr_F	Forward primer for amplification of apramycin resistance cassette from pSET152 with *Spe*I linker	GG*ACTAGT*AGGATCTTCACCTAG ATCCTT
Apr_R	Reverse primer for amplification of apramycin resistance cassette from pSET152 with *Spe*I linker	TT*ACTAGT*AACGTCATCTCGTTCT CCGCT
KatG_F	Forward primer for amplification of *M. tuberculosis katG* with *Swa*I linker	CTGGT*ATTTAAATA*GGCTGATCCA CCC
KatG_R	Reverse primer for amplification of *M. tuberculosis katG* with *Hpa*I linker	GGA*GTTAAC*TCAACCCGAATCAG C
4395_UP_F	Forward primer for amplification of 5' flanking region of *aac(2')* (i.e. *MAB_4395*) with *Nde*I linker	AAT*CATATG*GAACGAACCGACTT ATACACAGGCG
4395_UP_R	Reverse primer for amplification of 5' flanking region of *aac(2')* (i.e. *MAB_4395*) with *Nhe*I linker	AAT*GCTAGC*GACGGTTGGCATAT TGGACACAGC
4395_DW_F	Forward primer for amplification of 3' flanking region of *aac(2')* (i.e. *MAB_4395*) with *Nhe*I linker	AAT*GCTAGC*TACGAACTGGGGGC GTTGAGTG
4395_DW_R	Reverse primer for amplification of 3' flanking region of *aac(2')* (i.e. *MAB_4395*) with *Mlu*I linker	AAT*ACGCGT*GGTGTCCCTGACCA CCGAGCG
C_4395_F	Forward primer for amplification of *aac(2')* (i.e. *MAB_4395*) with *Hind*III linker for cloning into complementation vector	CG*AAGCTT*GTGGAAATTGTGATG TTGCC
C_4395_R	Reverse primer for amplification of *aac(2')* (i.e. *MAB_4395*) for cloning into complementation vector with endogenous *Hind*III site	GC*AAGCTT*GTCATCACCAGCCGT
P_4395_F	Forward primer for amplification of *aac(2')* (i.e. *MAB_4395*) hybridization probe	TATGACGGCCTCGATCTCGAAGA
P_4395_R	Reverse primer for amplification of *aac(2')* (i.e. *MAB_4395*) hybridization probe	ATCGAGATCCCAGATGACGGTG T
4124_UP_F	Forward primer for amplification of 5' flanking region of *eis1* (i.e. *MAB_4124*) with *Apa*I linker	AAA*GGGCCC*ATTCCCGTTCAACA TCCTGCCG
4124_UP_R	Reverse primer for amplification of 5' flanking region of *eis1* (i.e. *MAB_4124*) with *Nde*I linker	TAA*CATATG*GCCCAAATACCGCC CTAATTCTAACA
4124_DW_F	Forward primer for amplification of 3' flanking region of *eis1* (i.e. *MAB_4124*) with *Nde*I linker	AAA*CATATG*ACCCTCCTCCCACA CAACGC
4124_DW_R	Reverse primer for amplification of 3'	AAT*GCTAGC*TCACCGATCTGGAC

	flanking region of *eis1* (i.e. *MAB_4124*) with *Nhe*I linker	GAGGATG
P_4124_F	Forward primer for amplification of *eis1* (i.e. *MAB_4124*) hybridization probe	CTCAACGCGAATACATCTCG
P_4124_R	Reverse primer for amplification of *eis1* (i.e. *MAB_4124*) hybridization probe	GAATTCCACACTGCCGCTGG
4532c_UP_F	Forward primer for amplification of 5' flanking region of *eis2* (i.e. *MAB_4532c*) with *Hpa*I linker	AAGCGT*GTTAAC*GACGAGCAAAAAC
4532c_UP_R	Reverse primer for amplification of 5' flanking region of *eis2* (i.e. *MAB_4532c*) with *Pfl23*II and *Pac*I linker	AGCAT*CGTACG*AATGC*TTAATTAA*TCTGGAATGTGTTGTGAGCGA
4532c_DW_F	Forward primer for the amplification of 3' flanking region of *eis2* (i.e. *MAB_4532c*) with *Pac*I linker	GAAT*TTAATTAA*GCCACCCACTTTT
4532c_DW_R	Reverse primer for amplification of 3' flanking region of *eis2* (i.e. *MAB_4532c*) with *Pfl23*II linker	AATGC*CGTACG*GATGTCAACGCAGCCCCCAA
C_4532c_F	Forward primer for amplification of *eis2* (i.e. *MAB_4532c*) complementation fragment with *Hind*III linker	GC*AAGCTT*TCGCGACAACTAGAA
C_4532c_R	Reverse primer for amplification of *eis2* (i.e. *MAB_4532c*) complementation fragment with *Hind*III linker	CG*AAGCTT*CGGTCCACACAGTTGT
P_4532c_F	Forward primer for amplification of *eis2* (i.e. *MAB_4532c*) hybridization probe	CTAGAAGTCGTCGGGCGCAC
P_4532c_R	Reverse primer for amplification of *eis2* (i.e. *MAB_4532c*) hybridization probe	CGCCGCTGACGTGGATGTG

Table S2. MIC determination of *M. abscessus* deletion and complemented mutants

Strains	*M. abscessus* ATCC 19977				*Δaac(2')*				*Δaac(2')* pMV361-*aac(2')*				*Δaac(2')* pMV361				*Δeis1*				*Δeis2*			
Day of MIC reading	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12
Compound	MIC (mg/L)																							
Kanamycin B	4	8	16	16	0.125	0.125	0.25	0.25	data not reported*				data not reported*				4	8	16	16	1	2	2	4
Tobramycin	4	8	8	16	0.125	0.25	0.25	0.25	2	4	8	16	0.25	0.25	0.5	0.5	4	8	8	16	2	4	8	16
Dibekacin	8	16	32	32	0.5	1	2	2	8	8	32	32	0.5	1	2	2	8	16	32	32	8	16	32	32
Arbekacin	0.5	1	1	2	0.5	0.5	1	1	0.5	1	1	1	0.5	0.5	1	1	0.5	1	1	1	0.5	1	1	1
Gentamicin C	2	4	8	8	0.5	1	1	2	2	4	8	8	0.5	1	2	2	2	4	8	8	2	4	8	8
Isepamicin	0.5	1	2	2	0.5	1	1	2	0.5	1	2	2	0.5	1	2	2	0.5	1	2	2	<0.25	0.5	0.5	1
Amikacin	1	2	4	8	1	2	4	8	1	2	4	8	1	2	4	8	1	2	4	8	0.25	0.25	0.5	0.5
Kanamycin A	1	1	2	4	0.5	1	2	2	data not reported*				data not reported*				1	1	2	2	0.25	0.5	1	1
Apramycin	0.5	0.5	1	1	0.5	0.5	1	1	0.5	0.5	1	1	0.5	1	1	1	0.5	1	1	1	0.5	0.5	1	1
Streptomycin	32	32	64	64	32	32	64	64	32	32	64	64	32	32	64	64	32	32	64	64	32	32	64	64
Hygromycin B	128	256	>256	>256	128	256	>256	>256	128	256	>256	>256	128	256	>256	>256	128	256	256	>256	8	16	16	32
Capreomycin	64	128	256	>256	64	128	256	>256	64	256	256	>256	64	256	256	>256	64	256	256	>256	4	4	8	8

Strains	*Δeis2* pJB-*apr*-*eis2*				*Δeis2* - pJB-*apr*				*Δaac(2') Δeis1*				*Δaac(2') Δeis2*				*Δeis1 Δeis2*				*Δaac(2') Δeis1 Δeis2*			
Day of MIC reading	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12	3	5	7	12
Compound	MIC (mg/L)																							
Kanamycin B	data not reported*				data not reported*				0.063	0.125	0.125	0.25	0.063	0.125	0.125	0.25	1	1	2	4	0.063	0.13	0.13	0.25
Tobramycin	data not reported*				data not reported*				0.125	0.25	0.25	0.5	0.063	0.125	0.125	0.25	1	2	8	16	0.063	0.13	0.13	0.25
Dibekacin	data not reported*				data not reported*				1	2	2	2	1	1	2	2	4	16	16	32	0.5	1	2	2
Arbekacin	0.5	1	1	2	0.5	1	1	2	0.25	0.5	0.5	1	0.25	0.5	0.5	1	0.25	0.5	0.5	1	0.5	0.5	0.5	1
Gentamicin C	data not reported*				data not reported*				0.5	1	1	2	0.5	1	1	1	2	4	8	8	0.5	1	1	1
Isepamicin	0.5	0.5	2	2	<0.25	0.5	0.5	1	0.5	1	2	2	<0.25	0.5	0.5	1	<0.25	0.5	0.5	0.5	<0.25	0.5	0.5	0.5
Amikacin	1	2	4	8	0.25	0.25	0.5	0.5	1	2	4	8	0.25	0.25	0.5	0.5	0.13	0.25	0.25	0.5	0.25	0.25	0.25	0.25
Kanamycin A	data not reported*				data not reported*				0.5	1	2	2	0.25	0.5	0.5	0.5	0.25	0.5	0.5	1	0.25	0.25	0.5	0.5
Apramycin	data not reported*				data not reported*				0.5	0.5	1	1	0.5	0.5	0.5	1	0.5	0.5	1	1	0.5	0.5	0.5	0.5
Streptomycin	32	32	64	64	32	32	64	64	16	32	64	64	32	32	32	64	16	32	32	64	16	32	32	64
Hygromycin B	128	128	256	256	8	16	16	32	128	256	256	>256	8	16	16	32	8	16	16	32	8	16	16	16
Capreomycin	64	128	128	256	4	4	8	8	64	128	256	>256	4	4	8	8	4	4	8	8	4	4	8	8

*The presence of a resistance cassette in the vector backbone of the complementation vector interferes with drug susceptibility testing and MICs for these compounds are therefore not reported.

ADDENDUM

Personal contribution to chapter 2

My contribution as first author to this manuscript was as follows:

- Design of the study.
- Generation of all *M. abscessus* double and triple deletion mutants.
- Generation of *M. abscessus* Δ*eis2* pJB-*apr*-*eis2* and *M. abscessus* Δ*eis2* pJB-*apr* strains.
- Drug susceptibility testing of *M. abscessus* strains.
- Discussion of the results.
- Writing of the manuscript.
- Revision of the manuscript.

CHAPTER 3

Effect of β-lactamase production and β-lactam instability on MIC testing results for *Mycobacterium abscessus*

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ABSTRACT

Objectives: Limited treatment options available for *M. abscessus* infections include the parenteral β-lactam antibiotics cefoxitin and imipenem, which show moderate *in vitro* activity. Other β-lactam antibiotics (except meropenem) have no considerable *in vitro* activity, due to their rapid hydrolysis by a broad-spectrum β-lactamase (Bla_*Mab*). We here addressed the impact of β-lactamase production and β-lactam *in vitro* stability on *M. abscessus* MIC results and determined the epidemiological cut-off (ECOFF) values for cefoxitin, imipenem and meropenem.

Methods: By liquid-chromatography high-resolution mass spectrometry (LC-HRMS), we assessed the *in vitro* stability of cefoxitin, imipenem and meropenem. *M. abscessus* ATCC 19977 strain and its isogenic bla_{Mab} deletion mutant were used for MIC testing. Based on MIC distributions of *M. abscessus* clinical strains, we determined ECOFFs of cefoxitin, imipenem and meropenem.

Results: A functional Bla_*Mab* increased MICs of penicillins, ceftriaxone and meropenem. LC-HRMS data showed significant degradation of cefoxitin, imipenem and meropenem during standard antibiotic susceptibility testing procedures. MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are influenced by incubation time.

Conclusions: The results of our study support administration of imipenem, meropenem and cefoxitin, for treatment of patients infected with *M. abscessus*. Our findings on *in vitro* instability of imipenem, meropenem and cefoxitin explain the problematic correlation between *in vitro* susceptibility and *in vivo* activity of these antibiotics and question the clinical utility of susceptibility testing of these chemotherapeutic agents.

INTRODUCTION

Mycobacterium abscessus is a dreadful and arduous to treat mycobacterial pathogen with high-level innate resistance to most commercially available antibiotics, including the antituberculous agents.[1-5] Clinically relevant cases are predominantly associated with pulmonary infections in patients with cystic fibrosis or bronchiectasis and disseminated disease in immunocompromised individuals.[2-4] *M. abscessus* is also highly resistant to disinfectants and therefore, it ordinarily causes skin and soft tissue infections following plastic surgery, tattooing or body piercing.[3,5-8] Several healthcare-associated outbreaks of *M. abscessus* infections, that have been reported worldwide, highlight the increasing medical importance of this multidrug-resistant pathogen and the urgent need for reliable medication strategies.[3-4,9]

As *M. abscessus* clinical isolates are uniformly resistant to standard chemotherapeutic agents, so far, no reliable antibiotic regimen for *M. abscessus* pulmonary infections has been established. Antibiotic administration is empirical and heavily relies on *in vitro* antibiotic susceptibility testing (AST) by broth microdilution and definitive subspecies identification.[3,10] The clinical importance of *M. abscessus* subspecies identification (*M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) is attributed to the fact that, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii,* have a functional inducible erythromycin ribosome methyltransferase gene [*erm(41)*] which confers macrolide resistance, whereas *M. abscessus* subsp. *massiliense* has a nonfunctional *erm(41)* leading to macrolide susceptibility and thereby, *M. abscessus* subsp. *massiliense* infections have more favorable prognosis and treatment outcomes.[10-18] The American Thoracic Society (ATS) recommends for the treatment of *M. abscessus* lung infections a combination therapy of an oral macrolide (clarithromycin or azithromycin) for clinical isolates susceptible to macrolides and the intravenous aminoglycoside drug amikacin, administered together with a parenteral β-lactam antibiotic, cefoxitin or imipenem.[3,19-21] However, with the exception of the macrolide class, very limited data are present in the literature concerning the correlation between AST and the clinical efficacy of these recommended antimycobacterial drugs.[22] The clinical relevance of AST remains a controversy, particularly on account of technical problems associated with AST methods, reproducibility of AST results, significant discrepancies between *in vitro* susceptibility and *in vivo* activity of a given drug, solubility and stability issues of the drugs used.[23] Ideally, clinicians could take advantage of AST for *M. abscessus,* when the *in vitro* susceptibility to a drug is consistent with a clinically achievable

drug exposure *in vivo*, resulting in favourable treatment outcomes. Accordingly, clinical susceptibility breakpoints (CBP) must represent minimal inhibitory concentration (MIC) distributions and zone diameter distributions of wild-type and resistant strains, resistance mechanisms, dosing regimens, drug pharmacokinetics, pharmacodynamics and epidemiological cut-off values (ECOFFs) and must allow prediction of treatment outcomes.[22-25] The Clinical and Laboratory Standards Institute (CLSI) is the only organization worldwide that has published AST guidelines for rapid growing mycobacteria (RGM)[24] recommending susceptibility testing of amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline or minocycline, imipenem, linezolid, moxifloxacin, trimethoprim-sulfamethoxazole and tobramycin.[25] The breakpoint concentrations for these antibiotics have nonetheless been set on the basis of *in vitro* driven studies and their efficacy (except the new drugs linezolid and tigecycline) has been clinically evaluated by Wallace *et al.* principally in patients with extrapulmonary disease.[26] To date, the European Committee on Antimicrobial Susceptibility Testing (EUCAST), has not officially announced guiding principles for nontuberculous mycobacteria (NTM) susceptibility testing.

Frequently used therapeutics for *M. abscessus* infections are the parenteral β-lactam antibiotics cefoxitin and imipenem[24], which have moderate *in vitro* activity, with MIC values reported by Dubée *et al.* for *M. abscessus* subsp. *abscessus* CIP 104536 of 32 and 8 mg/L, respectively.[27] Meropenem, an ultrabroad-spectrum carbapenem closely related to imipenem[28], displayed a lower MIC value (16 mg/L) than cefoxitin against *M. abscessus* subsp. *abscessus* CIP 104536.[27] Most of the other β-lactam antibiotics have no considerable *in vitro* activity due to their rapid hydrolysis by a broad-spectrum class A β-lactamase, encoded by *MAB_2875*, namely Bla_*Mab*, which was reported as the major determinant of β-lactam resistance in *M. abscessus*.[27,29] Determination of the kinetic parameters of this enzyme revealed that it can slowly hydrolyze imipenem and meropenem, while cefoxitin hydrolysis by Bla_*Mab* is immensely slow, as the methoxy group at cefoxitin's β-lactam ring was predicted to block the activity of class A β-lactamases.[27,29-30]

As imipenem, meropenem and cefoxitin are known to have limited *in vitro* stability[31], we assessed by liquid-chromatography high-resolution mass spectrometry (LC-HRMS) the *in vitro* stability of these β-lactams and by exploiting a bla_{Mab} deletion mutant that we generated, we addressed the biological effect of β-lactam stability and β-lactamase production on MIC testing results after different periods of incubation. The direct impact of β-lactam stability on AST testing was further addressed by MIC determination of fresh and pre-incubated β-lactam

antibiotics towards the ampicillin susceptible *Escherichia coli* XL1-Blue MRF' strain. What is more, based on MIC distributions of *M. abscessus* clinical strains, mainly isolated from respiratory samples, we estimated ECOFFs for cefoxitin, imipenem and meropenem. Our results show that MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are severely influenced by stability issues, thereby questioning the clinical utility of AST towards cefoxitin, imipenem and meropenem, but not the use of these antibiotics in patients with *M. abscessus* infections.

MATERIALS AND METHODS

Bacterial strains and growing conditions

(i) *Escherichia coli* strains were cultivated in Luria-Bertani (LB) medium or on LB agar plates, when necessary, containing either ampicillin (120 mg/L) or apramycin (50 mg/L), at 37 °C, overnight. For *E. coli* MIC testing and all cloning steps when constructing the bla_{Mab} deletion vector pSE-*katG-aac(3)IV*-Δ*MAB_2875*, *E. coli* XL1-Blue MRF' (Stratagene, Switzerland) was used. (ii) *Mycobacterium abscessus* strains were grown in Middlebrook 7H9-OADC-Tween 80 liquid medium or on LB agar plates, when needed, containing either apramycin (50 mg/L) or isoniazid (32 mg/L), at 37 °C for 5 days. The *M. abscessus* ATCC 19977 type strain, *M. abscessus* Δbla_{Mab} mutant strain and 62 clinical isolates of *M. abscessus* (33 *M. abscessus* subsp. *abscessus*, 17 *M. abscessus* subsp. *bolletii* and 12 *M. abscessus* subsp. *massiliense* isolates) were used throughout this study. The *M. abscessus* clinical isolates were mainly isolated from respiratory samples that were received at the Swiss National Center for Mycobacteria within the years 2007-2014.

Definitive subspecies identification of clinical isolates

Subspecies identification of *M. abscessus* isolates (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*) was based on the 16S rRNA, *rpoB* and *erm(41)* gene sequences.[12-14,32-33] Subspecies attribution for *M. abscessus* complex isolates was performed according to Tortoli *et al*.[12] The obtained sequences were analyzed with the use of Lasergene SeqMan software (DNASTAR, USA), the SmartGene IDNS mycobacteria and *rpoB* databases (SmartGene, Switzerland), and the BLAST algorithm (http://blast.ncbi.nlm.nih.gov).

Antibiotics

Imipenem, meropenem, cefoxitin, ceftriaxone, ceftazidime, penicillin G, ampicillin, amoxicillin, amikacin, apramycin and isoniazid were bought from Sigma-Aldrich, Switzerland. All compounds were dissolved in H_2O, according to the manufacturer's recommendations, were filter sterilized, aliquoted into stock solutions of 3 to 50 g/L and finally stored at -20 °C.

Deletion of *MAB_2875* in *M. abscessus*

An 1.4-kbp *Hpa*I/*Pfl23*II fragment from position 2926650 to 2928011 (5′ bla_{Mab} flanking sequence) and an 1.5-kbp *Pfl23*II/*Pfl23*II fragment from position 2928642 to 2930147 (3′ bla_{Mab} flanking sequence) were PCR amplified using genomic DNA from *M. abscessus* ATCC 19977 (5′-GAATTAGTTAACCAGGTGTGATCCAGATGCCCG-3′, 5′-GAATTTCGTACGGGCCGCCGAAATCCTTTTCC-3′ and 5′-GAAATACGTACGCCATCGTGATGGCGGTACTCAC-3′, 5′-GAATTTCGTACGGTGTCTACCAGTCCTTGCACACC-3′, respectively) and cloned into the pSE-*katG-aac(3)IV* vector with corresponding enzymes, resulting in the bla_{Mab} deletion vector pSE-*katG-aac(3)IV*-Δ*MAB_2875*. *M. abscessus* bla_{Mab} deletion mutant was generated using a double selection strategy described previously in detail by Rominski *et al*.[34-35] In brief, pSE-*katG-aac(3)IV*-Δ*MAB_2875* was transformed into electrocompetent *M. abscessus* ATCC 19977. 100 µL competent cells were mixed with 1-2 µg plasmid DNA (supercoiled) and electroporated in a BioRad Gene pulser II using 4 mm gap electroporation cuvettes and the settings: 2.5 kV, 1000 Ohms and 25 µF.[36] Cells were resuspended after electroporation in 0.9 mL of 7H9-OADC-Tween 80 liquid medium and incubated for 5 h at 37 °C. Proper dilutions were eventually plated on selective agar and after 5 days of incubation, single colonies were picked and restreaked on LB agar plates with appropriate antibiotics. Transformants were selected on LB agar plates containing apramycin and identified by *aac(3)IV* colony PCR (primers: 5′-CACCTTCTTCACGAGGCAGACCTC-3′ and 5′-GGTCTGACGCTCATGGAACTAGTAGG-3′). Isolation of genomic DNA was performed by phenol-chloroform-isoamyl alcohol extraction as described previously[37] and single crossover transformants were confirmed by Southern blot analysis with a 0.45-kbp *Sac*I 3′ bla_{Mab} DNA probe and subjected to counter-selection on LB agar plates containing isoniazid. Single colonies were screened for deletion of *MAB_2875* by PCR (primers: 5′-

GTACACCGTCTTCGGGACG-3′ and 5′-GAAAGTGCGAGTACGCGTCTG-3′) and their genotype was finally verified by Southern blot analysis using the same 0.45-kbp *Sac*I 3′ bla_{Mab} DNA probe. In this way, a 0.63-kbp region of the *MAB_2875* was deleted from the chromosome of *M. abscessus* ATCC 19977.

Nitrocefin test

For the detection of β-lactamase production in *M. abscessus* ATCC 19977 type strain and the Δbla_{Mab} mutant strain we performed chromogenic nitrocefin test (Becton Dickinson, USA). Tubes containing (i) pure nitrocefin solution, (ii) nitrocefin solution inoculated with 5 colonies of *M. abscessus* ATCC 19977 type strain and (iii) nitrocefin solution inoculated with 5 colonies of *M. abscessus* Δbla_{Mab} mutant, were incubated for 1 h at room temperature before a photo was taken.

MIC assays

MIC assays were performed with the microdilution method and according to the CLSI guidelines,[25] but with incubation of the 96-well microtiter plates at 37 °C, as described previously.[34-35] Directly after the preparation of the antibiotic stock solutions, working solutions were prepared by diluting the corresponding stock solutions in Cation-Adjusted Müller-Hinton Broth (CAMHB; pH 7.4) (Becton Dickinson, Switzerland) to a concentration of 512 mg/L (final antibiotic concentration used for AST; 256 mg/L) and were subsequently stored at -80 °C. Shortly before the conduction of a MIC experiment (day 0), working solutions were thawed for the first time and were directly used. Alternatively, working solutions were thawed and pre-incubated for 1 to 7 days at 37 °C and their inhibitory effect was compared to freshly thawed antibiotics. Two-fold serial dilutions of the freshly thawed (and pre-incubated: for the pre-incubation MIC experiments) working solutions were prepared using CAMHB in sterile 96-well microtiter plates (Greiner Bio-One, Switzerland). Each 96-well microtiter plate included a positive growth control lacking antibiotic and a sterile negative control containing only CAMHB. To achieve a final inoculum titer of 1-5 x 10^5 cfu/mL, while final volume per well was 100 μL, bacterial suspensions of strains with smooth or rough phenotypes were adjusted to a McFarland standard of 0.5 and 3.0, respectively, and subsequently diluted in CAMHB. The proper titer of the inocula was confirmed by obtaining cfu counts on LB agar plates. MIC values were assessed by visual inspection after incubation

at 37 °C for 16 h for *E. coli* strains and 3, 5, 7 and 12 days for *M. abscessus*. MIC assays towards *E. coli*, *M. abscessus* ATCC 19977 and *M. abscessus* bla_{Mab} deletion mutant were conducted in triplicates. MIC assays towards *M. abscessus* complex clinical isolates were performed once.

LC-HRMS assays

In vitro antimicrobial stability of cefoxitin, imipenem and meropenem was evaluated using CAMHB and the maximum final antibiotic concentration used for AST (256 mg/L). A different test tube was prepared for each antibiotic and for every reading time point, i.e. 3, 5, 7 and 12 days. A day 0 control was used to define the 100% relative antibiotic concentration. The antibiotic containing test tubes were incubated under the same temperature conditions as the microtiter plates used for AST (37 °C). Cefoxitin, imipenem and meropenem were quantified by liquid-chromatography high-resolution mass spectrometry (LC-HRMS) on a Q Exactive hybrid instrument (Thermo Fisher, Switzerland). Samples were precipitated using a precipitation solution consisting of methanol/acetonitrile 80/20 v/v, containing the corresponding stable-isotope labelled internal standards. After centrifugation at 11.700 x g for 10 min at 4 °C, 10 µL of the clear supernatant were injected into the turbulent flow online extraction system. As extraction column, a Cyclone column (50 x 0.5 mm) and for analytical chromatography, a Hypersil Gold C8 column (100 x 3 mm), were used. Mobile phases consisted of 10 mM ammonium acetate in water + 0.1% formic acid and 10 mM ammonium acetate in methanol/acetonitrile 50/50 v/v + 0.1% formic acid. Samples were analyzed in positive heated electrospray ionization mode and detection was done in full-scan mode with a resolution of 70000 full width at half maximum (calculated for m/z 200).

ECOFF determination

MIC data from 62 *M. abscessus* complex clinical isolates were collected and the resistance population analysis charts were calculated using the integrated histographical analysis tool of Microsoft Excel. Median MIC_{50} values of the *M. abscessus* complex clinical strains against cefoxitin, imipenem and meropenem were calculated using SPSS software. All ECOFF values were determined by the eye-ball method.[38-39]

RESULTS AND DISCUSSION

Generation of *M. abscessus MAB_2875* deletion mutant

Similarly to our previously published techniques[34-35], we intended to generate an *M. abscessus* Δ*MAB_2875* mutant, which would enable us to directly address the role of the β-lactamase production of *M. abscessus* on MIC testing results for β-lactam antibiotics. The bla_{Mab} deletion mutant was constructed by transformation of *M. abscessus* ATCC 19977 with plasmid pSE-*katG-aac(3)IV*-Δ*MAB_2875* applying apramycin positive selection[40] and a *katG*-dependent isoniazid counterselection strategy (Figure 1a).[34-35] Deletion of *MAB_2875* was confirmed by Southern blot analysis (Figure 1b). While this study was ongoing, a Δ*MAB_2875* mutant was also generated by Dubée *et al.*[27], but with different cloning, genetic manipulation and selection strategies.

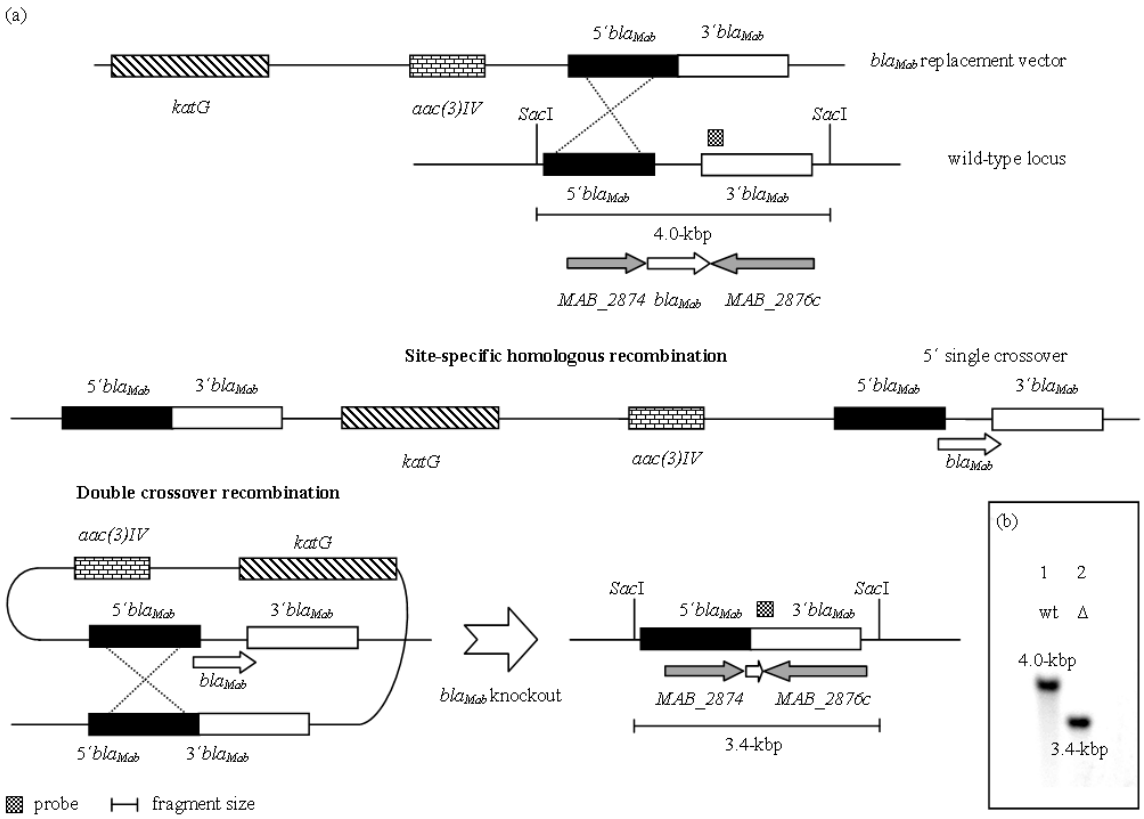

Figure 1. Genotypic analyses of *M. abscessus* bla_{Mab} locus. (a) Schematic drawing of genotypes and recombination events. (b) Southern blot analysis confirmed the deletion of *MAB_2875* from the genome of *M. abscessus*. Genomic DNA of *M. abscessus* ATCC 19977 (1) and *M. abscessus* transformant with pSE-*katG-aac(3)IV*-Δ*MAB_2875* targeting vector after KatG-dependent isoniazid-counterselection (2) was digested with *Sac*I and probed with a 0.45-kbp fragment from the 3′ bla_{Mab} flanking region. Based on *M. abscessus* genome

annotation and vector sequence, the pattern is consistent with hybridization to a 4.0-kbp fragment of the wild-type (wt) parental strain and to a 3.4-kbp fragment of the *M. abscessus* Δbla_{Mab} mutant (Δ).

M. abscessus bla_{Mab} deletion mutant is deficient in β-lactamase production

To assess the β-lactamase production of *M. abscessus* Δbla_{Mab} mutant, we performed a chromogenic nitrocefin test. The cephalosporin nitrocefin changes colour from yellow to red, in the presence of β-lactamases, that hydrolyze the amide bond in its β-lactam ring.[41] As Figure 2 shows, the tube containing yellow pure nitrocefin solution did not change colour. The tube containing nitrocefin solution inoculated with *M. abscessus* ATCC 19977, changed colour and became red, while the tube containing nitrocefin solution inoculated with the *M. abscessus* Δbla_{Mab} mutant stayed yellow, confirming that the *M. abscessus* bla_{Mab} deletion mutant is deficient in β-lactamase production. These results align with the findings from Dubée *et al.*[27], who reported Bla_*Mab* as the single determinant of β-lactam resistance in *M. abscessus.*

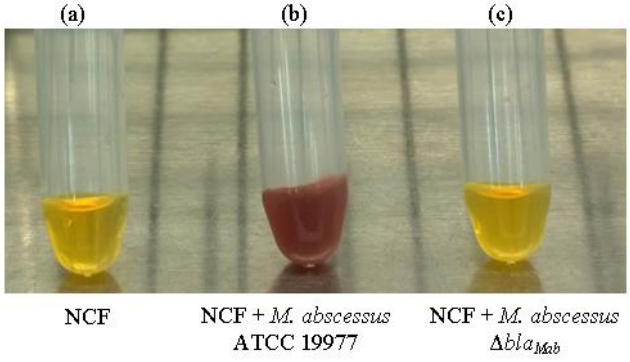

Figure 2. Chromogenic nitrocefin solution (NCF) test results after incubation for 1 h at 37 °C. (a) Tube containing pure nitrocefin solution. (b) Tube containing nitrocefin solution inoculated with *M. abscessus* ATCC 19977 wild type. (c) Tube containing nitrocefin solution inoculated with *M. abscessus* Δbla_{Mab} mutant.

AST of *M. abscessus* Δ*MAB_2875*

In order to explore the role of Bla_*Mab* in *M. abscessus* β-lactam resistance, MIC values of *M. abscessus* ATCC 19977 type strain and *M. abscessus* Δbla_{Mab} mutant were determined with the microdilution method for the β-lactam antibiotics, imipenem, meropenem, cefoxitin, ceftriaxone, ceftazidime, penicillin G, ampicillin, amoxicillin, and a control antibiotic from the class of aminoglycosides, i.e. amikacin (Table 1). As formerly reported[27], *M. abscessus* ATCC 19977 was highly resistant to all β-lactam antibiotics tested except imipenem,

meropenem and cefoxitin. Deletion of *bla*$_{Mab}$ significantly reduced the MIC values of all penicillins tested (penicillin G, ampicillin, amoxicillin) and the cephalosporin ceftriaxone. MIC values of ≥256 mg/L of the oxyimino-cephalosporin ceftazidime towards both *M. abscessus* ATCC 19977 and *M. abscessus* Δ*bla*$_{Mab}$ mutant suggest that ceftazidime is not active against *M. abscessus.* Cefoxitin MIC values of 32 mg/L (day 3) for both the *M. abscessus* ATCC 19977 and Δ*bla*$_{Mab}$ mutant (Table 1) agree with earlier reported data[27], corroborating previous molecular modeling and structural analyses[42] which delineated that substitutions of the β-lactam ring by a methoxy group in cefoxitin inhibits the activity of class A β-lactamases.[30] As expected, amikacin MICs were independent of the *bla* genotype.

Table 1. AST results of *M. abscessus* wildtype and Δ*bla*$_{Mab}$ mutant*

Strains	*M. abscessus* ATCC 19977				*M. abscessus* Δ*bla*$_{Mab}$			
Day	3	5	7	12	3	5	7	12
Antibiotic	MIC (mg/L)							
Imipenem	2	4	8	64	1	2	4	64
Meropenem	8	16	32	256	2	4	4	16
Cefoxitin	32	32	64	128	32	32	32	128
Ceftriaxone	64	64	64	64	8	16	16	16
Ceftazidime	>256	>256	>256	>256	256	>256	>256	>256
Penicillin G	>256	>256	>256	>256	2	4	4	4
Ampicillin	>256	>256	>256	>256	4	4	8	8
Amoxicillin	>256	>256	>256	>256	2	4	4	4
Amikacin	1	2	4	8	1	2	4	8

*Broth microdilution method

To date, none of the recent studies on β-lactam susceptibility testing towards *M. abscessus* CIP 104536 and *M. abscessus* Δ*bla*$_{Mab}$ mutant has reported MIC data obtained after 3 days of incubation.[27,29-30,43-44] Indeed, CLSI guidelines[25] for imipenem MIC testing against RGM recommends maximum incubation period of 3 days and state that "the reported breakpoints for imipenem are considered tentative". Our observation that imipenem, meropenem and cefoxitin MIC values rise substantially with the incubation time for both the *M. abscessus* ATCC 19977 strain and the *M. abscessus* Δ*bla*$_{Mab}$ mutant (Table 1), in correlation with the fact that certain antibiotics undergo partial degradation under *in vitro* testing conditions[45], led us to further investigate the effect of the β-lactam stability on MIC testing procedures.

Effect of β-lactam stability on *E. coli* MIC testing

In order to experimentally determine whether the results of the *M. abscessus* MIC testing against β-lactams after 3, 5 and 7 days of incubation clearly reflect β-lactamase production or are affected by instability of the antibiotics tested, we performed a MIC experiment using *E. coli* XL1-Blue MRF' model strain, that produces no β-lactamases, against fresh and pre-incubated (at 37 °C, for 1 to 7 days) β-lactam antibiotics. In that way, all MIC results could be obtained after 16 h of incubation of the microtiter plates and putative MIC differences between the fresh and the pre-incubated antibiotics could be directly correlated to instability of the tested antibiotics. Our results show that pre-incubation at 37 °C affects MIC results of imipenem, meropenem and to a lesser extend cefoxitin and ceftazidime (Figure 3a; 3b), whereas the MIC results of ceftriaxone, penicillin G, ampicillin, amoxicillin and as expected the control stable aminoglycoside antibiotic amikacin, are not influenced by pre-incubation (Figure 3b; 3c; 3d). By assuming that drug degradation follows an exponential decay process and using the data displayed in Figure 3, we calculated the half-life values of imipenem as 0,75 day, of meropenem as 1,5 days and of cefoxitin as 3 days. We therefore conclude that the increased MIC values of the pre-incubated antibiotics, already before day 3, reflect instability of the antibiotics. Reading of *M. abscessus* MICs at day 3 and later gives a de facto misleading impression for the true extend of activity of imipenem, meropenem and cefoxitin.

***In vitro* antimicrobial stability of cefoxitin, imipenem and meropenem by LC-HRMS**

Based on our results suggesting that incubation of cefoxitin, imipenem and meropenem at 37 °C gives rise to misleading MIC results, we intended to address the *in vitro* stability of these β-lactams, by quantifying them using LC-HRMS. Tubes containing CAMHB and the maximum antibiotic concentration used for AST (256 mg/L) were incubated at 37 °C. The 100% relative antibiotic concentration of each antibiotic was defined by a day 0 control tube. At days 3, 5, 7 and 12 the concentrations of cefoxitin, imipenem and meropenem were quantified. Data obtained by LC-HRMS method, clearly show degradation of cefoxitin, imipenem and meropenem. Particularly, the relative concentration at day 3 for imipenem dramatically dropped to 3.3%, whereas for cefoxitin and meropenem it dropped to 36.6 % and 35.6 %, respectively (Figure 4e; 4j; 4o). By using the results of this assay, the calculated half-life of imipenem was 0.6 days, for meropenem 2 days and for cefoxitin 2.1 days. These findings agree with those deducted from the *E. coli* MIC testing and confirm that the MIC

values of cefoxitin, imipenem and meropenem against *M. abscessus* at day 3 and later are misleading, as concentration of the active compound is lowered. Especially for imipenem, MIC_{50} at day 3 was 16 mg/L (Figure 4j). However, growth inhibition of half of the clinical isolates was in reality achieved by considerably lower drug amounts, as the true active relative concentration of imipenem shrank to 3.3% by day 3 due to the short half-life of this antibiotic. Consequently, due to instability of imipenem, meropenem and cefoxitin, *M. abscessus* MIC determination after 3 days of incubation does not reflect the potency of these three compounds, especially since clinical administration[3] of these drugs is daily and in multiple doses. These data, on the one hand, question the utility of susceptibility testing of imipenem, meropenem and cefoxitin, but on the other hand support administration of these parenteral β-lactam antibiotics as part of combinational regimens for the treatment of *M. abscessus* infections.

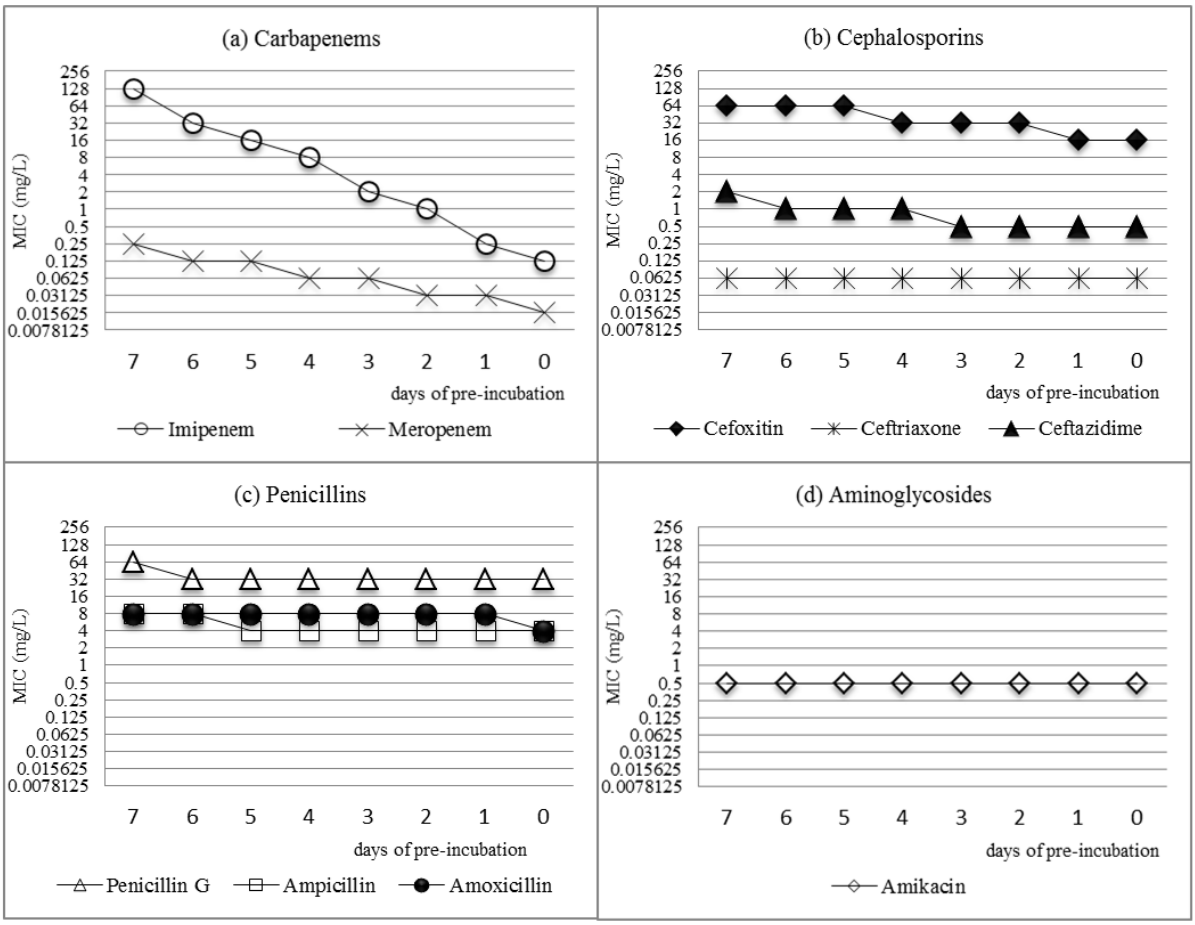

Figure 3. MIC values of *E. coli* XL1-Blue MRF' strain against fresh and pre-incubated (at 37 °C, for 7 to 1 days) β-lactam antibiotics. (a) Carbapenems: imipenem and meropenem. (b) Cephalosporins: cefoxitin, ceftriaxone and ceftazidime. (c) Penicillins: penicillin G, ampicillin and amoxicillin. (d) Aminoglycoside (control): amikacin.

ECOFF values of cefoxitin, imipenem and meropenem for *M. abscessus* complex

According to EUCAST, for the determination of CBP values and improvement of the MIC interpretation for clinical isolates, ECOFFs need to be defined to separate the wild-type population from any non-wild-type strains with acquired drug resistance mechanisms to the chemotherapeutic agents in question.[46] We, therefore, endeavoured to estimate ECOFF values for cefoxitin, imipenem and meropenem by visual inspection of the MIC histographic distribution analysis (eyeball method)[36-37] of 62 *M. abscessus* complex clinical strains isolated from respiratory samples, after 3, 5, 7 and 12 days of incubation at 37 °C (Figure 4). The results show that MIC distributions of our collection of *M. abscessus* complex isolates towards cefoxitin, imipenem and meropenem are close to the breakpoints established by CLSI and that the eyeball-estimated ECOFF values did not show a separation of the *M. abscessus* complex isolates into two distinct subgroups; wild-type and resistotype. ECOFFs were set at the highest MIC value observed among the isolates tested and all of these isolates are therefore classified in the wild-type subgroup. According to this classification, a putative clinical isolate with an additional β-lactam resistance mechanism would have a very high MIC value (higher than the corresponding ECOFF) that would be neither detected by standard AST laboratory procedures nor relevant for clinical interpretation. Interestingly, the presence or absence of a functional or non-functional β-lactamase in *M. abscessus* could not be predicted by the MIC distribution of the clinical isolates, as the MIC values determined for the *M. abscessus* Δbla_{Mab} (Table 1) fall in the MIC distribution of the "wild-type" subgroup of clinical isolates towards all three β-lactams (Figure 4).

Furthermore, our results show that MIC and MIC_{50} values of cefoxitin, imipenem and meropenem are greatly influenced by incubation time; the longer the incubation period, the higher the MIC of the individual strains, the MIC_{50} and the estimated ECOFF values (Figure 4). The presented MIC distribution bars, as the MIC_{50} and ECOFF lines, would represent valid data for MIC, MIC_{50} and ECOFF values, only if the drugs were 100% stable. However, according to the LC-HRMS results, cefoxitin, meropenem and particularly imipenem were proven as unstable compounds and thereby, MIC values of the *M. abscessus* complex clinical isolates towards these three β-lactams at day 3 and later are certainly overestimated, as already discussed. If the concentration of the biological active form of the drug could have been kept constant during the AST procedures, we expect that the position of the MIC distribution bars, MIC_{50} and ECOFF lines would be shifted to the left in all MIC distribution analysis graphs (Figure 4). Our observations highlight the impact of cefoxitin, imipenem and

meropenem stability issues on MIC, MIC_{50} and ECOFF determination for *M. abscessus* complex clinical isolates.

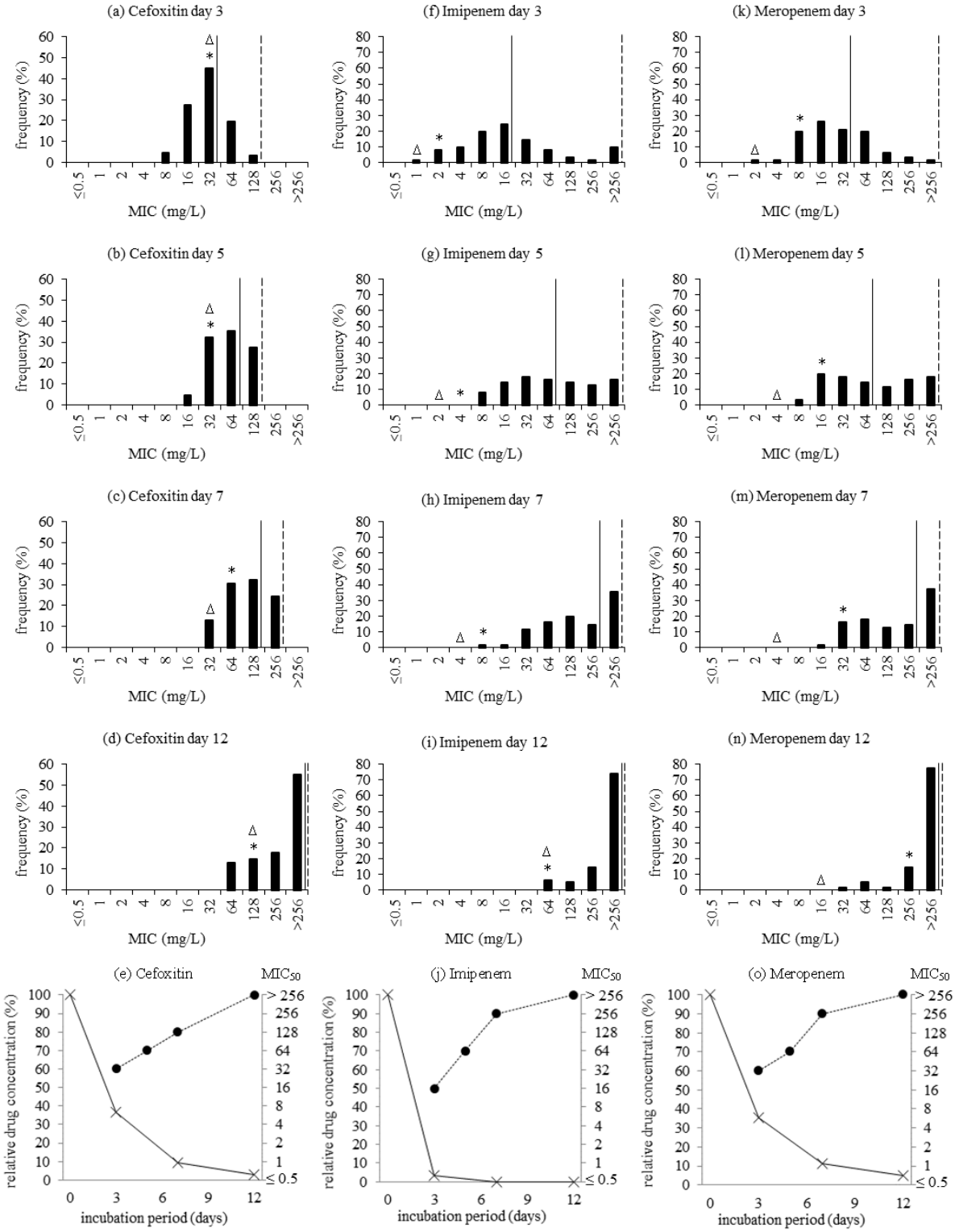

Figure 4. MIC distributions for cefoxitin (a-d), imipenem (f-i) and meropenem (k-n) for *M. abscessus* complex strains (n = 62). MIC_{50} values are indicated by a black vertical continuous line, whereas ECOFF values are indicated by a black vertical broken line. Symbol "*" shows MIC values of *M. abscessus* ATCC 19977 type strain, while symbol "Δ" shows MIC values of *M. abscessus* Δbla_{Mab} mutant. Drug stability of cefoxitin (e),

imipenem (j) and meropenem (o) in comparison with median MIC_{50} values. Crosses connected with a black continuous line show relative drug concentrations and filled circles connected with a black broken line show MIC_{50} values.

Conclusions

Taken together, our study confirmed Bla_*Mab* as the major determinant of innate β-lactam resistance in *M. abscessus* and addressed experimentally the biological effect of β-lactamase production and β-lactam stability on *M. abscessus* MIC testing results after different incubation periods. Our results show that MIC, MIC_{50} and ECOFF values of cefoxitin, imipenem and meropenem are immensely influenced by incubation time. LC-HRMS data and MIC determination of pre-incubated drugs for the fast growing model organism (*E. coli*) proved significant degradation of cefoxitin, imipenem and meropenem during standard AST procedures, explaining the problematic correlation between *in vitro* susceptibility of these three β-lactams and their *in vivo* activity. Our findings critically question the clinical utility of cefoxitin, imipenem and meropenem susceptibility testing, but further support administration of these chemotherapeutic agents for treatment of *M. abscessus* infections.

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ADDENDUM

Personal contribution to chapter 3

My contribution as first author to this manuscript was as follows:

- Design of the study.
- Generation of *M. abscessus* bla_{Mab} deletion mutant.
- Nitrocefin test of *M. abscessus* bla_{Mab} deletion mutant.
- Drug susceptibility testing of *M. abscessus* ATCC 19977 and its isogenic bla_{Mab} deletion mutant.
- Pre-incubation MIC experiments to study the effect of β-lactam stability on *E. coli* MIC testing
- Discussion of the results.
- Writing of the manuscript.
- Revision of the manuscript.

OUTLOOK

Based on the findings of this study, there are several questions and lines of research arising which should be pursued:

- Design, synthesis and *in vitro* testing of novel compounds that can escape Arr_*Mab*-mediated rifamycin resistance in *M. abscessus*.

- Study the structural similarities between Arr_*Mab* and domain III of *Pseudomonas aeruginosa* exotoxin A. Does Arr_*Mab* play a role in *M. abscessus* virulence?

- The *M. abscessus* Δ*eis1* mutant did not show any phenotype and therefore does not contribute to AG resistance, at least not during *in vitro* growth. Overexpression of Eis1, phenotypical analyses and biochemical assays could address the physiological and enzymatic function of Eis1.

- Through a biochemical approach, the exact residues of specific aminoglycoside antibiotics that Eis2 acetylates could be identified.

- As streptomycin MICs in the mutant strains remained at the high wild-type MIC levels, streptomycin resistance mechanisms in *M. abscessus* should be further studied.

- This study demonstrated that apramycin, arbekacin, isepamicin and kanamycin A exhibit excellent *in vitro* activities against *M. abscessus* ATCC 19977 type strain and that the activity of these drugs is not affected by the AAC(2') and Eis proteins. MIC testing of these aminoglycosides against a broader set of *M. abscessus* clinical isolates should be performed. The obtained data may provide invaluable basic knowledge for designing clinical trials aiming at implementation of improved treatment regimens against *M. abscessus*.

- Further studies addressing the problematic correlation between *in vitro* susceptibility of imipenem, meropenem and cefoxitin and their *in vivo* activity should be performed.

- Finally, since genetic manipulation of *M. abscessus* is now well-advanced, additional *M. abscessus* antibiotic resistance mechanisms, e.g. fluoroquinolones, ethionamide, pyrazinamide, could be investigated.

PUBLICATIONS

2017 **Anna Rominski**, Anna Roditscheff, Petra Selchow, Erik C. Böttger, Peter Sander. Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591. *J Antimicrob Chemother* 2017; 72: 376-384.

2017 **Anna Rominski**, Petra Selchow, Katja Becker, Juliane K. Brülle, Michael Dal Molin, Peter Sander. Elucidation of *Mycobacterium abscessus* aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes. *J Antimicrob Chemother* 2017; 72: 2191-2200.

2017 **Anna Rominski**, Bettina Schulthess, Daniel M. Müller, Peter M. Keller, Peter Sander. The effect of β-lactamase production and β-lactam instability on MIC testing results of *Mycobacterium abscessus. J Antimicrob Chemother* 2017; Epub ahead of print. doi:10.1093/jac/dkx284.

PRESENTATIONS

2016 74th Annual Assembly of the Swiss Society of Microbiology (SSM), Bern, Switzerland:

Poster presentation

Deciphering aminoglycoside resistance mechanisms of Mycobacterium abscessus

Anna Rominski*, Petra Selchow, Juliane K. Brülle, Katja Becker, Anna Roditscheff, Erik C. Böttger and Peter Sander

2016 9th Microbiology and Immunology (MIM) Ph.D. Program Retreat, Grindelwald, Switzerland:

Oral presentation

Deciphering the rifamycin resistance mechanisms of an antibiotic nightmare – Mycobacterium abscessus

Anna Rominski*, Anna Roditscheff, Petra Selchow, Erik C. Böttger and Peter Sander

*Presenting Author

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2013-2017 **Ph.D. studies** at the Institute of Medical Microbiology, University of Zurich, Switzerland, under supervision of Prof. Dr. Peter Sander.

Characterization of antibiotic resistance genes from Mycobacterium abscessus

2010-2012 **M.Sc. studies** in "Molecular Biology, Genetics Applications and Diagnostic Markers", Department of Biochemistry and Biotechnology, University of Thessaly, Greece. (With Honours).

Study of the interactions between E6 oncoprotein and its cellular targets in infected cells with Human Papilloma Virus -16 and -18

2005-2009 **4-year B.Sc. (+Diploma) studies** in Biology with specialization in Molecular Biology, Molecular Genetics and Development at School of Biology, Faculty of Sciences, Aristotle University of Thessaloniki, Greece. (With Honours).

Molecular and genetic characterization of symbiotic bacteria in natural populations of the olive fly (*Bactrocera oleae*).

2002-2005 **4th High School** of Drama, Greece. Natural Sciences. (With Honours).